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PROCEEDINGS
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*STRAIN SPECIFICITY AND PRODUCTION OF ANTIBIOTIC SUBSTANCES. IX. BACTERIOSTATIC AND BACTERICIDAL PROPERTIES OF NEOMYCIN AND DEVELOPMENT OF RESISTANT STRAINS**

By David Weiss and Selman A. Waksman

New Jersey Agricultural Experiment Station, Rutgers University

Communicated March 25, 1950

Introduction.—It was previously reported[1] that neomycin is highly effective against various bacteria, including both streptomycin-sensitive and -resistant strains. Further studies brought out the fact that when cultures of *Escherichia coli* were allowed to remain in contact with neomycin, no strains either highly resistant to or dependent on this antibiotic were produced. A certain degree of resistance to neomycin could be developed, but this took place rather slowly; the resistant cells also showed a marked tendency to become sensitive again. The growth of such resistant strains in the presence of neomycin in concentrations slightly lower than those required for inhibition was poor and was accompanied by marked changes in cell morphology.

The purpose of this investigation was threefold: first, to study the bactericidal and bacteriostatic action of neomycin, especially as compared to streptomycin; second, to measure the development of resistance to neomycin among sensitive bacteria; and third, to establish any possible synergistic effects of neomycin with streptomycin. The development of resistance of an organism to an antibiotic is of considerable importance in determining its usefulness as a potential chemotherapeutic agent. In measuring the potential synergistic combinations of the two antibiotics, the minimum concentrations which give the maximum antibacterial effect are first determined. The ratios of these antibiotics are then so adjusted that the addition of a minimum amount of neomycin would cause the optimum antibacterial action of streptomycin, without allowing the development of bacterial resistance to this antibiotic.

Experimental.—A laboratory strain of *E. coli* (No. 54) was used in these studies. This culture was initially sensitive to 3 μg./ml. of streptomycin

and to 0.5 u/ml. of neomycin. Higher concentrations of either antibiotic completely inhibited growth in nutrient broth or on nutrient agar. All cultures were incubated at 37°C.

Bacteriostatic and Bactericidal Properties of Neomycin.—As in the case with other antibiotics, the size of inoculum influenced considerably the amount of neomycin required for growth inhibition. When a 24-hr. agar slant culture of *E. coli* was washed with isotonic saline solution, and heavy suspensions of bacteria were added to nutrient agar containing different concentrations of the antibiotics, the amount of neomycin required for complete inhibition could be increased from 0.5 u/ml. to 5 u/ml. Similar concentrations of streptomycin had no effect upon such heavy inocula.

In comparing the bactericidal action of neomycin with streptomycin, suspensions of *E. coli* in 10-ml. portions of broth, to give approximately 180,000 cells per milliliter, were treated with varying concentrations of the two antibiotics. After 14 and 28 hours of incubation, aliquot portions were removed and plated out on nutrient agar (table 1). Complete in-

TABLE 1

BACTERIOSTATIC AND BACTERICIDAL ACTION OF NEOMYCIN AND STREPTOMYCIN

Inoculum—180,000 cells/ml. of broth. Plate method used for making counts of viable bacteria

ANTIBIOTIC PER ML. OF BROTH	NUMBER OF VIABLE CELLS PER ML., AFTER INCUBATION FOR 14 HRS.	28 HRS.
Control	800,000,000	700,000,000
Streptomycin, 1 μg.	340,000,000	330,000,000
Streptomycin, 5 μg.	80,000,000	40,000,000
Streptomycin, 20 μg.	0	0
Neomycin, 1 u	5,000	1,500,000
Neomycin, 5 u	0	0
Neomycin, 20 u	0	0

hibition of growth as well as a marked bactericidal effect took place with 20 μg./ml. of streptomycin. A concentration of 1 μg./ml. had only a slight inhibiting effect, and 5 μg./ml. had a marked bacteriostatic action but only a limited bactericidal effect. Neomycin was bacteriostatic and bactericidal in concentration of 5 u/ml. and with 1 u/ml., although with the latter not all the bacteria were killed. There was apparently some adaptation of the bacteria to the lowest concentration of neomycin, since there was an increase in the number of cells on further incubation of the cultures.

In the following experiment, turbidimetric measurements were made. A known suspension of bacteria was introduced into sterile Klett-Sommerson tubes. Known volumes of medium containing the antibiotic in different concentrations were added. A zero reading was taken immediately for each tube, and the tubes were incubated. The inoculum was obtained

by washing 24-hr. nutrient agar slants of *E. coli* with saline solution. The antibiotic solutions were heated at 70°C. for 10 minutes for sterilization.[2] Here again neomycin was found (table 2) to have a far greater bactericidal effect than streptomycin, 1.25 u/ml. of the first being comparable to 5 μg./ml. of the second.

A study was made next of the effect of neomycin upon a streptomycin-resistant strain of *E. coli*. The strain was repeatedly streaked on nutrient agar plates containing streptomycin and neomycin in different concentrations. It was found to give "normal" growth on all concentrations of the

TABLE 2

EFFECT OF NEOMYCIN AND STREPTOMYCIN UPON THE GROWTH OF *E. coli*

Turbidimetric readings. Original inoculum 2000 cells/ml. of broth.

ANTIBIOTIC PER ML. OF BROTH	INCREASE IN TURBIDITY OVER ZERO READINGS, AFTER INCUBATION IN			
	1 DAY	3 DAYS	7 DAYS	14 DAYS
Control	50	224	325	435
Streptomycin, 1.25 μg.	18	79	119	229
Streptomycin, 2.5 μg.	22	78	106	206
Streptomycin, 5 μg.	0	0	0	0
Streptomycin, 10 μg.	0	0	0	0
Neomycin, 1.25 u	0	0	0	0
Neomycin, 2.5 u	0	0	0	0
Neomycin, 5 u	0	0	0	0

TABLE 3

EFFECT OF NEOMYCIN UPON STREPTOMYCIN-RESISTANT STRAIN OF *E. coli*

Turbidimetric readings. Original inoculum 1000 cells/ml. of broth.

ANTIBIOTIC PER ML. OF BROTH	INCREASE IN TURBIDITY OVER ZERO READING, AFTER INCUBATION IN	
	5 DAYS	12 DAYS
Control	162	284
Streptomycin, 5 μg.	141	269
Streptomycin, 10 μg.	162	365
Streptomycin, 100 μg.	146	275
Neomycin, 1.25 u	0	0
Neomycin, 2.5 u	0	0
Neomycin, 5 u	0	0

first and was sensitive to 1.25 u/ml. of the second. A suspension of a 24-hr. nutrient agar slant was used to inoculate a series of Klett tubes. The inoculum was so adjusted as to give 1000 cells/ml. of the final medium. Growth was more rapid and much heavier in the tubes containing streptomycin than in the control tube (table 3), possibly because of the presence of streptomycin-dependent cells in the culture. The lowest concentration of neomycin, 1.25 u/ml., completely inhibited growth of this strain. These results tend to show that streptomycin-resistant strains of *E. coli* do not develop simultaneous resistance to neomycin.

Development of Resistance by E. coli to Neomycin.—The *E. coli* culture used in these experiments was inhibited by 1 u/ml. of neomycin or less, the size of inoculum being of great importance in this connection, as shown in table 4. On several occasions, a few colonies appeared on the plates containing 3 to 5 u/ml. of neomycin, and even on a 10 u/ml. plate. The cultures obtained from these colonies died, however, after passage through liquid medium containing 12 u/ml. of neomycin. The only time when such colonies grew in neomycin-containing media in concentrations exceeding 2 u/ml. was when the plates were streaked with a heavy cell suspension. No growth was ever observed on dilution plates or in liquid media containing more than 2.5 u/ml. of neomycin.

A streptomycin-dependent strain of *E. coli* failed to grow in the absence of streptomycin or in the presence of neomycin. A streptomycin-sensitive strain of *B. subtilis* was completely inhibited by 0.2 u/ml. of neomycin.

TABLE 4

GROWTH OF *E. coli* IN VARIOUS CONCENTRATIONS OF NEOMYCIN AS AFFECTED BY SIZE OF INOCULUM

NUMBER OF CELLS IN INOCULUM	NEOMYCIN (U/ML.)						
	0	0.2	0.4	0.6	0.8	1.0	1.8
	Turbidity[a]						
63.7×10	30	19	0	0	0	0	0
63.7×10^2	34	22	0	0	0	0	0
63.7×10^3	35	21	29[b]	0	0	0	0
63.7×10^4	30	18	30[b]	6[b]	0	0	0
63.7×10^5	31	18	24	28[c]	22[b]	0	0
63.7×10^6	30	23	19	20	16	0	0

[a] 100 per cent transmission. Readings represent averages of three tubes at 144 hrs. incubation except where noted.

[b] Average turbidity of two tubes only.

[c] Turbidity of one tube only.

A neomycin-resistant strain of *Mycobacterium* 607 grew at fairly high concentrations of neomycin; it showed also sporadic resistance to streptomycin, although in most cases it was completely inhibited by 1 μg./ml.

In a natural population, a few cells resistant to neomycin were found occasionally (table 5). The most resistant cells were grown in the presence of increasingly higher concentrations of this antibiotic. Considerable difficulty was encountered, however, since such resistant cultures again became readily sensitive to neomycin. When cultures were made resistant to concentrations of neomycin as high as 70 u/ml. by gradual transfer, they failed, after several transfers, to make further growth or grew only in media containing no neomycin.

A long period of incubation was sometimes required for growth of cultures in neomycin-containing media. Growth often failed to appear in cultures incubated at 28°C. Yeast extract agar media gave growth in some instances when none was obtained on beef extract media.

A strain of *E. coli* was finally developed which was resistant to 100 u/ml. of neomycin. Fifteen consecutive transfers were required for this purpose, using increasing concentrations of the antibiotic. Agar slants containing 50, 60 and 70 units/ml. of neomycin were streaked with this strain. Growth was obtained after one day's incubation at 37°C. The slants were stored in the icebox, together with cultures growing in media containing no neomycin. After four weeks, all the cultures were tested. Only those which grew on media containing no neomycin contained viable cells; this was also true of a few slants containing less than 30 units/ml. neomycin. The cultures growing in neomycin-free media were still resistant to 40 u/ml. *E. coli* can thus be made resistant to neomycin in concentrations less than 100 u/ml., but the strain dies out rapidly or becomes sensitive when exposed for prolonged periods to neomycin. The strain can retain its resistance to neomycin when kept on neomycin-free media.

TABLE 5

DEVELOPMENT OF NEOMYCIN-RESISTANT CELLS OF *E. coli* IN THE ABSENCE OF NEOMYCIN

INCUBATION, HRS.	TOTAL NUMBER OF CELLS/ML.	CELLS RESISTANT TO 5 UNITS NEOMYCIN/ML. AGAR
0	3,480,000	1
4	13,800,000	22
8	116,000,000	40
12	184,000,000	77
24	310,000,000	48
48	392,000,000	8
72	363,000,000	8
96	367,000,000	2
144	322,000,000	4
219	327,000,000	5

The rate of growth and the number of cells were less in the neomycin-resistant than in the sensitive cultures, even upon prolonged incubation. Growth on solid media was always in the form of very small, moist, glistening colonies; large colonies or heavy growth was seldom obtained, such growth being sensitive to neomycin. Growth in liquid media showed a fine granular dispersion rather than the homogeneous turbidity of young cultures of sensitive strains. Microscopic appearance revealed cells normal in size, but darker in staining, during the first 20 hrs. of incubation; after that, the cells usually became elongated and had a granular appearance. Those slants which initially grew in the presence of neomycin and then died out showed a lysed appearance, the presence of growth being almost indiscernible to the naked eye.

In the early stages of the development of neomycin resistance, no increase in resistance to streptomycin was noted. When the neomycin-resistant strain was kept on neomycin-free media in the icebox for four weeks

and then tested, it was found to show an increase in resistance to 75 μg./ml. of streptomycin. This points to the possibility of a certain amount of overlapping in the mode of action of the two antibiotics.

The Synergistic Effects of Neomycin and Streptomycin.—For the study of the synergistic effects of the two antibiotics on *E. coli*, both turbidimetric measurements and determination of numbers of viable cells by the agar plate method were used. Certain combinations of neomycin with streptomycin showed marked bacteriostatic and bactericidal effects, which were considerably greater than the corresponding concentrations of either antibiotic alone. This was even true when the ratio of streptomycin to neomycin was 15:1 and higher, complete inhibitions of growth taking place with ratios of 6:1.

A combination of 1 μg./ml. of streptomycin and 0.2 u/ml. of neomycin exerted a bactericidal effect, though 3.2 μg./ml. of streptomycin alone or 0.2 u/ml. of neomycin alone showed relatively little action. This is brought out in table 6. The agar plate method makes possible a further insight into

TABLE 6

SYNERGISTIC ACTION OF NEOMYCIN AND STREPTOMYCIN UPON SMALL NUMBERS OF *E. coli* CELLS

Turbidimetric readings. Inoculum 1750 cells/ml. of broth

ANTIBIOTIC PER 1 ML. OF BROTH	INCREASE IN TURBIDITY OVER ZERO READING AFTER INCUBATION IN 2 DAYS	4 DAYS	7 DAYS
Control	205	312	372
Streptomycin, 1.2 μg.	59	88	133
Streptomycin, 3.2 μg.	45	60	75
Neomycin, 0.2 u	42	52	90
Neomycin, 0.5 u	0	0	0
1 μg. streptomycin + 0.2 u neomycin	0	0	0
3 μg. streptomycin + 0.2 u neomycin	0	0	0

the inhibiting action of the two antibiotics upon the growth of *E. coli:* A combination of 2.5 μg./ml. streptomycin plus 2.5 u/ml. of neomycin gave nearly complete inhibition, whereas heavy growth was obtained on the plates containing either of these antibiotics alone, as well as on the plates containing 5 μg./ml. of streptomycin (table 7).

To test the possible development of resistance of the surviving cells, transfers were made from the few colonies that appeared on the plates which contained both antibiotics into nutrient broth to which varying concentrations of streptomycin, neomycin, and combinations of both were added. No resistance to neomycin greater than one unit was found. The colonies isolated from the plates containing both antibiotics showed no great resistance to streptomycin either.

Summary.—1. The bacteriostatic and bactericidal properties of neomycin were compared with those of streptomycin. Growth of *E. coli* in nutrient broth was measured by the turbidimetric and plating procedures.

2. The bactericidal action of neomycin was much greater than that of streptomycin. Concentrations as low as 1 u/ml. of neomycin greatly inhibited growth of *E. coli* in heavy suspensions, whereas similar concentrations of streptomycin had little effect. When the inoculum was so adjusted that the final medium contained a few hundred or thousand cells per milliliter, concentrations of neomycin as low as 0.5 u/ml. inhibited growth completely.

3. A streptomycin-resistant strain of *E. coli* showed no increase in resistance to neomycin. A streptomycin-dependent strain of *E. coli* failed to grow in the presence of neomycin.

4. *Mycobacterium* 607 was inhibited by 1 u/ml. of neomycin. A strain of this organism made neomycin-resistant was usually inhibited completely by 1 μg./ml. of streptomycin, but in several instances colonies were found on plates containing 50 μg./ml.; this strain remained resistant to high concentrations of neomycin for several months.

5. A strain of *E. coli* originally sensitive to neomycin was made resistant to 100 u/ml. of neomycin. Such cultures usually died out or became sensitive again after several transfers. A few cultures retained their resistant properties to a greater extent when grown in media containing no neomycin. The incubation time of neomycin-resistant cultures was longer than that of sensitive cultures.

6. The neomycin-resistant strain of *E. coli* grew on solid media in the form of small, glistening colonies; in liquid media, growth appeared as a granular suspension. Dead colonies had a lysed appearance.

TABLE 7

SYNERGISTIC EFFECTS OF NEOMYCIN AND STREPTOMYCIN UPON *E. coli*, AS MEASURED BY AGAR PLATE METHOD

ANTIBIOTIC PER ML. OF AGAR	NUMBER OF COLONIES PER PLATE
Control	> 1000
Streptomycin, 2.5 μg.	> 300
Steptomycin, 5 μg.	46–> 300
Streptomycin, 10 μg.	0
Neomycin, 1.25 u	> 300
Neomycin, 2.5 u	33–119
Neomycin, 5 u	0
2.5 μg. streptomycin + 2.5 u neomycin	0[a]
3 μg. streptomycin + 2 u neomycin	0[a]
4 μg. streptomycin + 1 u neomycin	0–5[b]

[a] A single colony found on 2 out of 5 plates.
[b] A total of 7 colonies on 5 plates.

7. Certain combinations of neomycin and streptomycin showed considerably greater bacteriostatic and bactericidal effects than the corresponding concentrations of either antibiotic alone or concentrations of streptomycin equal to the total number of units of both antibiotics. Such antibacterial effects were never greater, however, than concentrations of neomycin equal to the total units of the two antibiotics. Very small amounts of neomycin in synergistic mixtures with streptomycin had a much greater effect than streptomycin alone.

8. No marked increase in the resistance of *E. coli* cells which survived exposure to neomycin and streptomycin was observed toward either antibiotic or combinations of the two.

9. The neomycin-resistant strain of *E. coli* showed some resistance to streptomycin after several weeks on media containing neither antibiotic.

10. Neomycin appears to be about four times as active, on a unit basis, as streptomycin against many bacteria. In view of the fact that the most potent neomycin preparations so far obtained have 250 units per milligram, the antibacterial potency of the two antibiotics is about similar on a gram basis.

Acknowledgment.—The authors wish to express their appreciation to Dr. Warren P. Iverson, formerly of this department, who carried out preliminary studies on this problem.

* Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Microbiology.

[1] Waksman, S. A., Lechevalier, H. A., and Harris, D. A., *J. Clin. Invest.*, **28**, 934–939 (1949).

[2] The neomycin was a crude preparation containing 100 u/mg; the streptomycin was in the form of sulfate assaying 650 μg./mg.

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*ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES. VII. CLITOCYBE ILLUDENS**

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Communicated March 16, 1950

Clitocybe illudens[1] was reported earlier from this laboratory[2] to evidence antibacterial activity. In later tests the antibacterial action of this fungus was found to be substantially greater on a corn steep agar medium than on the original thiamine-peptone agar or on a potato-dextrose agar. Grown on corn steep agar and tested by the agar disc method,[2, 3] zones of inhibition up to 25 mm. in diameter were observed for *Staphylococcus aureus*

and up to 40 mm. in diameter for *Mycobacterium smegma*. There was little or no effect on *Escherichia coli*.

Culture liquids with antibacterial activity were produced by growing the fungus at 25°C. on a corn steep medium in Fernbach flasks as previously described.[4] After three to four weeks the mycelial mats covered the surface and the culture liquids had an activity of from 64 to 256 dilution units per ml. against *Staph. aureus*, *Klebsiella pneumoniae* and *Myco. smegma*. Little inhibitory action was noted on *Bacillus mycoides*, *Bacillus subtilis*, *E. coli* or *Pseudomonas aeruginosa*. It was possible to obtain active liquid two weeks after reflooding such mats with fresh corn steep medium. Mats were successfully reflooded several successive times.

Isolation of Crystalline Substances.—Two antibacterial substances and one substance inactive for the organisms tested were isolated in crystalline form from culture liquids of *C. illudens*.

Extraction of the active material from the culture liquid with organic solvents was incomplete. However, the active material was adsorbed practically quantitatively by charcoal from which it could be eluted by aqueous acetone.

Bioassay of the material extracted by organic solvents and that left in the aqueous phase suggested the presence of more than one active component. Thus after extraction of culture liquid with chloroform the activity for *Myco. smegma*, compared to that for *Staph. aureus*, was increased in the organic solvent and decreased in the aqueous phase. By countercurrent distribution between chloroform and water, of the material eluted from the charcoal, three distinct components were separated.

Batches of culture liquid at their original pH of from 4.2 to 4.5 were stirred for an hour with 20 g. of Norit A (Pfanstiehl) per liter, using an efficient mechanical stirrer. The solution was usually kept in a cold room at 8° to 10°C. overnight, and the charcoal allowed to settle. After the charcoal had been filtered off by suction, through a thin layer of celite, a perfectly clear solution was obtained which had only a small percentage of the original activity; this was discarded. The Norit A, on which the active material was adsorbed, was stirred for three half-hour periods with 80 per cent acetone, the first time with 10 per cent of the volume of culture liquid used, then twice more with 5 per cent each time. The eluate was concentrated under reduced pressure, in a water bath at about 60°C. to remove the acetone, leaving about a liter of aqueous concentrate. This contained essentially all the active material originally present in the culture liquid. Fractionation was accomplished by countercurrent distribution. The distribution was carried out in ten funnels, using equal volumes of chloroform and water.

By concentration of the extract to a small volume under reduced pressure, and finally to a sirup under a stream of nitrogen, crystalline ma-

terial was usually obtained from "Chloroform 1" (the first chloroform extract which passed through the series of funnels) and sometimes also from "Chloroform 2." This material was over a hundred times as active against *Myco. smegma* as against *Staph. aureus;* it is designated *illudin M*. Chloroforms 4 to 10 on evaporation yielded inactive crystalline material.

"Water 1" (the original eluate after being washed with ten portions of chloroform) and "water 10" (the tenth water washing of the chloroform) were of low antibacterial potency and accounted for only a small fraction of the activity of the original culture liquid. These extracts were discarded. Waters 2 to 9 were extracted with two and one-half times their volume of ethyl acetate in five equal portions. On evaporation of the ethyl acetate, a crystalline compound highly active against both *Staph. aureus* and *Myco. smegma* was obtained. This substance is referred to as *illudin S*.

The yield of crystalline illudin S per liter of culture fluid (average of ten batches) was 0.33 g.; of crystalline illudin M, 0.04 g. About one-third of the activity of the original culture liquid against *Staph. aureus* was recovered in the crystalline material and about one-half of the activity against *Myco. smegma*. The average results per liter for ten batches of culture liquid are given below:

	WEIGHT, G.	*Staph. aureus* DILUTION UNITS	*Myco. smegma* DILUTION UNITS
Illudin S	0.33	75,460	20,650
Illudin M	0.04	260	30,140
Total	0.37	75,720	50,790
Culture liquid	...	230,000	101,000

By redistribution of mother liquors from which crystals had been obtained, more of each of the compounds was crystallized. The chloroform mother liquors yielded varying amounts of crystals of the inactive compound in addition to illudin M. Average results of redistribution of three batches of chloroform mother liquors and four batches of ethyl acetate mother liquors are given below:

		ACTIVITY	
	WEIGHT, G.	*Staph. aureus* DILUTION UNITS	*Myco. smegma* DILUTION UNITS
No. 1, Solids in $CHCl_3$ mother liquors	29.00	238,000	4,500,000
Illudin M from No. 1	1.01	6,200	907,000
Inactive crystals from No. 1	0.50	...	...
No. 2, Solids in ethyl acetate mother liquors	30.00	3,900,000	1,400,000
Illudin S from No. 2	5.51	718,000	804,000
Total illudin M and illudin S	6.52	724,200	1,711,000

If the additional material obtained by redistribution is taken into account the percentage of the activity of the original culture liquid against

Staph. aureus which was recovered in crystalline form was about 45 per cent; against *Myco. smegma*, about 80 per cent. While it is possible that other antibiotic substances may have been present, it is believed that illudin M and illudin S account for the major part of the activity of the culture liquids.

Chemical Characteristics of Illudin M and Illudin S.—The antibacterial activity of the culture liquid, or of the active crystalline compounds dissolved in water, was not reduced by bringing the solution to a quick boil. This method of sterilization was used in preparing samples for bioassay. The activity of the culture liquid was not affected by incubation for one hour at 37°C. and pH levels of from 3 to 11. While no systematic studies of stability have been made, it has been observed that the crystalline compounds are unstable under somewhat more stringent conditions than those mentioned.

Both active compounds are fairly soluble in organic solvents, less so in water. Illudin M is soluble in water to the extent of at least 1 mg. per ml.; illudin S is somewhat more soluble.

The three crystalline compounds have been further purified and characterized; the data given below suggest that they are probably of related molecular structure. The molecular formula of illudin M differs from that of illudin S by the elements of one molecule of water.

Compound	Illudin M	Illudin S	Inactive crystals
Recrystallized from	Aqueous ethanol	Acetone	Chloroform
Melting point (uncorr.)	130–131°	124–125°	72–74°
Absorption maxima ($m\mu$) in 95% ethanol	230, 320	235, 328	None between 215 and 360
$(\alpha)_D^{25}$ in absolute ethanol	−126	−165	−107
Analytical values, C, H[a]	C, 72.81 H, 8.05	67.78 8.16	60.39 8.29
Empirical formula, and calculated C, H	$C_{15}H_{20}O_5$ C, 72.53 H, 8.12	$C_{15}H_{22}O_4$ 67.62 8.33	$C_8H_8O_2$ 59.98 8.06
Molecular weight (found)[a]	241	264	204
Molecular formula	$C_{15}H_{20}O_5$ (mol. wt. 248)	$C_{15}H_{22}O_4$ (mol. wt. 266)	$C_{16}H_{16}O_4$ (mol. wt. 200)[a] or $C_{18}H_{24}O_4$ (mol. wt. 300)

All three compounds are neutral. A further similarity is their behavior in acid solution, in which characteristic changes occur in their absorption spectra. The products resulting from this change, and their possible bearing on the structure of the antibiotic compounds, will be reported separately.

Antibacterial and Antifungal Activity.—The potency of the active crystalline compounds, after recrystallization, was determined for a

number of bacteria by serial dilution[7] and is given in the following table. The activity is expressed as the minimum inhibitory concentration in μg. per ml. (p = partial inhibition)

Bacterium	Illudin S	Illudin M
Bacillus mycoides	> 500	> 500
Bacillus subtilis	31 (16p)	500
Escherichia coli	> 500	> 500
Klebsiella pneumoniae	4	16 (8p)
Mycobacterium smegma	4 (2p)	1 (0.5p)
Mycobacterium tuberculosis[8] H37R	6	1
Pseudomonas aeruginosa	500 (125p)	> 500
Staphylococcus aureus	4	250

The potency of the antibacterial compounds against *Staph. aureus*, *Myco. smegma* and *K. pneumoniae* was not reduced after incubation at 37°C. for three hours with 5 per cent human blood in 0.9 per cent saline with beef extract.

The antifungal activity of illudin S and illudin M was measured by serial dilution in a peptone medium at pH 6. Spore suspensions of the fungi were used as inoculum.[9] *Trichophyton* was incubated at 30°C.; the others at 25°C. The fungi tested included *Aspergillus niger*, *Chaetomium globosum* (USDA 1042.4), *Gliomastix convoluta* (PQMD4c), *Memnoniella echinata* (PQMD1c), *Myrothecium verrucaria* (USDA 1334.2), *Penicillium notatum*, *Phycomyces Blakesleeanus* (plus strain), *Saccharomyces cerevisiae*, *Stemphylium consortiale* (PQMD41b), and *Trichophyton mentagrophytes*. As much as 250 μg. per ml. of illudin S did not inhibit the growth of these fungi.

The antifungal activity of illudin M was somewhat greater, though the difference in response between fungi was marked. A wide range of partial inhibition (p) was noted for some species. The minimum inhibitory concentration of illudin M in μg. per ml. was 16 for *Memnoniella echinata*, 16 (2p) for *Penicillium notatum*, 32 (1p) for *Chaetomium globosum*, 64 (32p) for *Trichophyton mentagrophytes*, 125 (8p) for *Myrothecium verrucaria*, 250 (32p) for *Aspergillus niger*, 250p to 32p for *Stemphylium consortiale*, 250p to 125p for *Saccharomyces cerevisiae*, 250p for *Phycomyces Blakesleeanus* and more than 250 for *Gliomastix convoluta*.

Toxicity.—In preliminary tests, using Carworth male white mice weighing on an average, 16 g., all mice were killed by illudin S at 15.6 mg. per kilo in from seven to twenty-two hours, and by illudin M within forty-four hours. Four groups of five mice each were used in the tests: one control group, and three groups receiving 0.25, 0.50 and 1.0 mg. per mouse, respectively. The test compound was dissolved in 0.5 ml. of 0.9 per cent saline solution, and injected into a tail vein. The controls received only saline solution.

Persons handling *Clitocybe illudens* or its products showed some sensitivity on contact, which manifested itself as a dermatitis of varying degrees of severity.

Hollande obtained an antibacterial substance, clitocybine, from the sporophores and mycelium of *Clitocybe gigantea* (*Fr. ex. Sow.*) *Quel. var. candida* (*Bres.*) *Heim*. The chemical properties given for clitocybine[10] distinguish it from illudin M and illudin S.

The sporophores of *Clitocybe illudens* are poisonous when eaten.[11] Clark and Smith[12] concluded from chemical and biological evidence that *C. illudens* had a muscarin-like effect. They were able to overcome the toxic action on frogs' hearts by atropin. Illudin M and S do not appear to be related to muscarin; it is, however, possible that the compounds found in sporophores of the fungus differ from those produced in the culture liquid by the mycelium.

* This investigation was supported in part by grants from the Commonwealth Fund and the Lillia Babbitt Hyde Foundation. Technical assistance by Jane Bancroft, Elayne Shapiro and Gabriele Eisemann is gratefully acknowledged.

[1] We are indebted to Ross W. Davidson, Division of Forest Pathology, U. S. Department of Agriculture for the culture of *Clitocybe illudens*, No. 72027-S in his collection.

[2] Robbins, W. J., Hervey, A., Davidson, R. W., Ma, R., and Robbins, W. C., *Bull. Torrey Botan. Club*, **72**, 165–190 (1945).

[3] Hervey, A., *Ibid.*, **74**, 476–503 (1947).

[4] Robbins, W. J., Kavanagh, F., and Hervey, A., these PROCEEDINGS, **33**, 171–176 (1947).

[5] The carbon and hydrogen analyses and molecular weight determinations were carried out by Joseph Alicino, Metuchen, N. J.

[6] The molecular weight of this compound is uncertain since it shows some signs of decomposition during the camphor melt.

[7] Kavanagh, F., *Bull. Torrey Botan. Club*, **74**, 303–320 (1947).

[8] Determinations for *M. tuberculosis* were made through the courtesy of Dr. Walsh McDermott and Dr. W. C. Robbins of the Cornell Medical College.

[9] Kavanagh, F., Hervey, A., and Robbins, W. J., these PROCEEDINGS, **35**, 343–349 (1949).

[10] Hollande, A. Charles, *Compt. rend. acad. sci. Paris*, **228**, 1758–1759 (1949).

[11] Fisher, O. E., in Kauffman, C. H., *The Agaricaceae of Michigan*, **1**, 825–875 (1918).

[12] Clark, Ernest D., and Smith, Clayton, S., *Mycol.*, **5**, 222–232 (1913).

UNILATERAL NUCLEAR MIGRATION AND THE INTERACTIONS OF HAPLOID MYCELIA IN THE FUNGUS CYATHUS STERCOREUS

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The researches of Kniep[1] in 1919 established the fact that, among certain Basidiomycetes, sexuality is governed by two pairs of independently segregating factors. In many of these fungi, the mycelium which develops from a single spore is haploid and when two compatible haploid mycelia grow together and fuse, diploid or dicaryotic mycelium is formed. Compatibility of haploid mycelia is determined, in the so-called "tetrapolar" species, by two pairs of independently segregating factors, *A–a* and *B–b*. Haploid mycelia *AB*, *ab*, *Ab* and *aB* are sexually compatible only in combinations which provide the full set of factors, *AaBb*.[2]

Many fungi are now known to exhibit this type of sexuality. The method by which diploid mycelia are produced following fusion of compatible haploid mycelia was first demonstrated by Buller.[3] He was able to show that when two compatible haploid mycelia fuse, nuclei migrate from each mycelium into the other and, as a result of this nuclear migration, both haploid mycelia become converted into diploid mycelia. Buller further showed that in this process of "diploidization" the rate of nuclear migration greatly exceeds the normal growth rate of either the haploid or the diploid mycelium.

Despite Buller's experiments and other subsequent studies there is, as yet, little knowledge of the mechanism of nuclear migration in the higher fungi. It is not known, for instance, what role is played by the two pairs of compatibility factors in the process of diploidization. The interactions of the mycelia of certain fungi would seem to suggest that there may be a different function for each of the two pairs of factors. For example, the "barrage" phenomenon studied by Vandendries and Brodie[4] in *Lenzites betulina* occurs only when two incompatible haploid mycelia are paired which contain different members of one pair of factors, in this species designated as *B–b*, and the same *A* or *a* factor.

Experimenting with *Coprinus lagopus*, Buller[3] showed that each haploid of a compatible pair undergoes diploidization. The nuclei migrate from both haploid mycelia into the mycelium of the partner. Certain exceptions to this type of regular diploidization have been observed. Dickson[5] reported that in some compatible pairings of haploid mycelia of *Coprinus sphaerosporus* only one of the haploids became diploid.

Attention was drawn by Brodie[6] in 1948 to this kind of diploidization in

Cyathus stercoreus. In some pairings of haploid mycelia ($AB \times ab$) the AB became diploid; the ab remained haploid. The term "unilateral diploidization" was applied by Brodie to the phenomenon, in reference to the migration of nuclei in one direction only (from ab mycelium into AB) to distinguish the situation from that commonly observed in which nuclei migrate from both mycelia of the compatible pair.

The present writer, in 1947, began an investigation of unilateral diploidization in the hope of throwing more light on the general problem of nuclear migration and the processes controlled by the mating type factors (see Brodie[6]). The present paper is a preliminary résumé of the chief results obtained.

Forty monospore cultures were obtained in 1949 from spores taken from one peridiole of *Cyathus stercoreus* grown in laboratory culture by Dr. Brodie.[6] Although similar results have been obtained from three other series of isolates (152 in all) from different fruit bodies, the observations recorded here and the conclusions drawn are based entirely on the study of the 1949 series. This is done because the assignment of genetic factors for mating types based on the results of pairings is arbitrary, and since it was not possible to keep cultures in a healthy condition for more than one year, it was not possible to cross different series as would be necessary in order to prove that mating types of different series were strictly comparable.

After being grown for three weeks, the haploid mycelia were paired in tubes in all possible combinations. Pairings on agar plates were made when it was necessary to study the interaction of mycelia more closely. The formation of diploid mycelium bearing true clamp connections was used as the criterion of sexual compatibility. From the results, the haploid mycelia were assigned to four mating types: AB, ab, Ab and aB.

When haploid mycelia were paired on agar plates, several types of interaction were observed, as shown in table 1. We may now consider each of these types of reaction in turn.

I. Combinations of Compatible Mycelia ($AB \times ab$ *and* $Ab \times aB$).—As shown in table 1, the diploidization in the majority of pairings of compatible mycelia was of the "regular" type. When two compatible haploids came into contact, hyphal fusions took place in the region of contact and diploid mycelium gradually appeared around the periphery of both haploid mycelia. Within 5–7 days both mycelia had become completely diploid.

Unilateral diploidization was found in some pairings representing each of the compatible combinations of mycelia. In unilateral pairings of $AB \times ab$ (pairings in which unilateral diploidization was manifest), the AB mycelium was always the acceptor of nuclei, i.e., it became diploid; the ab mycelium was always the donor of nuclei and remained haploid. Uni-

TABLE 1

INTERACTIONS OF FORTY MONOSPOROUS MYCELIA OF *Cyathus stercoreus* WHEN PAIRED IN ALL POSSIBLE COMBINATIONS

REACTION TYPE	GENOTYPES OF PAIRED MYCELIA	REACTION	TOTAL COMBINATIONS	NUMBER GIVING VISIBLE REACTION	NUCLEAR MIGRATION		
					REGULAR	UNILATERAL	NUMBER TESTED
I	(Compatible)						
	$AB \times ab$	Formation of diploid mycelium	100	100	61	39	100
	$Ab \times AB$	Formation of diploid mycelium	66	66	14	6	20
					NUMBER SHOWING NO NUCLEAR MIGRATION BEYOND DEMARKATION LINE		NUMBER TESTED
II	(Incompatible, *A* factors different)						
	$AB \times aB$	Demarkation line formed	50	49	25		25
	$Ab \times ab$	Demarkation line formed	120	117	15		15
					NUMBER SHOWING NUCLEAR MIGRATION		NUMBER TESTED
III	(Incompatible, *B* factors different)						
	$AB \times Ab$	Blotchy mycelium formed	120	119	19		20
	$AB \times ab$	Blotchy mycelium formed	50	49	10		10
	WITHIN MATING TYPES			NO. GIVING REACTION	NO. TESTED	COMPLETELY UNILATERAL WITH COMPATIBLES	NUMBER TESTED
IV	$AB \times AB$	Vigorous mycelium	45	30	30	8	10
	$ab \times ab$	Vigorous mycelium	45	6	6	Not completed	..
	$Ab \times Ab$	Vigorous mycelium	66	6	6	5	5
	$aB \times aB$	Vigorous mycelium	10	6	6	Not completed	..

lateral behavior was found less frequently in the $Ab \times aB$ combinations. However, when it occurred the Ab mycelium always behaved as acceptor and the aB as donor. Table 1 shows the frequency of these two types of diploidization.

Individual haploid mycelia of one mating type might display unilateral diploidization when paired with certain haploids of the compatible mating type but display regular diploidization with others. At present, no definite pattern has been observed by means of which one can predict whether or not a given haploid belonging to one mating type will behave regularly or unilaterally when paired with a particular compatible haploid. Individual haploids within one mating type do not appear to fall into definite groups in this respect.

When monosporous mycelia were stained, it was found that in the majority of them the hyphal tips were multinucleate, the young cells immediately behind the tip contained irregular numbers of nuclei and the older cells were uninucleate. However, the hyphal tips of some AB and some Ab monosporous mycelia had a larger than average number of nuclei and most of the older cells of such mycelia contained two nuclei each. These two nuclei did not lie as close to one another as do the members of a regular dicaryon in true diploid mycelium. Also, no evidence of conjugate division of the pairs of nuclei found in AB and Ab mycelia has yet been obtained.

In every example thus far investigated, the binucleate monosporous mycelium has shown unilateral diploidization when paired with certain compatible regularly uninucleate mycelia, and it has always behaved as an acceptor (see table 1). Conversely, every AB or Ab mycelium which displayed unilateral diploidization with some compatible mycelia has, when stained, proved to be binucleate.

II. Pairings of Incompatible Mycelia Having B or b Factors in Common ($AB \times aB$ and $Ab \times ab$).—In these pairings a dense white line of demarkation developed in the region of contact between the two haploids. Mycelium taken from this line and examined microscopically consisted of wide, irregular cells, some of which bore incomplete or false clamp connections similar to those found by Brunswik[7] and others in combinations of incompatible mycelia. Quintanilha[8] showed that mycelium bearing false clamp connections consists of some cells containing two nuclei and some containing only one. The false clamp connection is formed during the conjugate division of the nuclei of the binucleate tip cells.

The mycelium on either side of the demarkation line remained uninucleate (if originally so) and unchanged in appearance. When mycelium from either side of the line was paired with tester mycelia of each of the four mating types, it formed diploid mycelium only with the mating type with which it was originally compatible, indicating that nuclei had not mi-

grated beyond the demarkation line. The line reaction was seen in most $AB \times aB$ pairings and in most $Ab \times ab$, i.e., where the two mycelia had different A factors but possessed the B or b factor in common. The line reaction was not observed in other combinations (see table 1).

III. Pairings of Incompatible Mycelia Having A or a Factors in Common ($AB \times Ab$ *and* $aB \times ab$).—In these pairings the two haploids grew together without a demarkation line. The mycelia resulting from such unions presented a very uneven growth throughout as to texture and color, and they grew more slowly than unpaired haploid mycelia. The term "blotchy" has been used to refer to this type of reaction.

When mycelium was taken from either side of a "blotchy" culture and paired with tester mycelia, it formed true diploid mycelium with the mating types with which both haploids had been compatible before pairing, i.e., the "blotchy" mycelium had become heterocaryotic. In some of the pairings of this "blotchy" mycelium with compatible monosporous mycelia, diploidization was unilateral, the "blotchy" mycelium acting as acceptor. More frequently, diploid mycelium grew out only from the region of contact between the "blotchy" and the monosporous mycelia. When stained, the "blotchy" mycelium was found to contain two nuclei in each cell, loosely associated in pairs, and similar in this respect to the unilateral AB and Ab mycelium described above. "Blotchy" reaction occurred in most pairings of $AB \times Ab$ and $aB \times ab$ and has not, as yet, been found elsewhere.

Binucleate monosporous AB or Ab mycelia (those which had exhibited unilateral diploidization when paired with a compatible uninucleate partner) also gave the "blotchy" reaction themselves when paired with tester mycelia which contained different B or b factors and similar A or a factors, but frequently did not cause the mycelium paired with them to become "blotchy." The uninucleate partner remained uninucleate and compatible only with the mating type with which it was originally compatible. From this it can be seen that the tendency for non-migration of nuclei exhibited when monosporous mycelia were paired with compatible partners was also exhibited in pairings with incompatible partners.

IV. Pairings within Mating Types ($AB \times AB$, *etc.*).—When all possible combinations of AB haploids were made, a variety of interactions was observed. When two uninucleate AB mycelia were paired which had never shown unilateral diploidization with any ab mycelium, they gave rise to a tufted, irregularly binucleate mycelium. When this binucleate mycelium was paired with any ab haploid, it invariably exhibited unilateral diploidization and acted as the acceptor.

When a uninucleate regular AB mycelium was paired with a binucleate AB which was usually unilateral, the union produced a very coarse, vigorous mycelium which resembled diploid mycelium except for the lack of

true clamp connections and of color usually present in the true diploid of *Cyathus stercoreus*. Some cells of this mycelium were binucleate; others were uninucleate and bore false clamp connections. When this mixed *AB* mycelium was paired with *ab* mycelium, unilateral diploidization usually resulted and the *AB* mycelium acted as the acceptor. Similar results were obtained from other pairings within other mating types.

Discussion and Conclusions.—The results given above indicate that, in this series of monosporous mycelia derived from one fruit body of *Cyathus stercoreus*, the pair of factors *B–b* control nuclear migration. When two incompatible mycelia differing in these factors but containing the same *A* or *a* factor were paired, nuclear migration occurred and heterocaryotic "blotchy" mycelium developed. In combinations of incompatible mycelia which had the same *B* or *b* factors, extensive nuclear migration did not take place.

The pair of factors *A–a* would appear to be more closely involved in the establishment of the dicaryon. When two incompatible mycelia differing in these factors were paired, a mycelial line developed between the two. Mycelium taken from this line bore false clamp connections, indicating that nuclei of the two paired mycelia had entered a cell and formed a temporary dicaryon. No nuclear migration beyond this line took place.

However, other factors may also be involved in the compatibility reaction. Since some nuclei of the heterocaryotic mycelia $AB \times Ab$ and $aB \times ab$ were found in pairs, differences other than those of the *A–a* factors must, in these cases, be responsible for the attraction between two nuclei. Furthermore, since nuclear migration and the formation of false clamp connections occurred in pairings within a single mating type, these factors must segregate independently of mating type.

Whenever unilateral diploidization occurred, the acceptor mycelium was binucleate. This binucleate condition may have arisen in the spore or in the mycelium within the first three weeks after germination. It has been shown that it can be produced by pairing two different haploids of the same mating type or two haploids of different mating types in which the *B–b* pair of factors is present but only one of the *A–a* pair is present. In these cases, the two nuclei within a binucleate cell must be genetically different. This difference may be responsible, in some way, for the nonmigration of nuclei.[9]

Each of the two commonly recognized pairs of compatibility factors apparently controls a different aspect of the diploidization process in *Cyathus stercoreus*. It is, however, also possible that certain functions are partially controlled by modifying factors located at different loci. Further analysis of these factors will be the object of later investigations.

Acknowledgment.—The writer wishes to express her gratitude to Dr. H. J. Brodie for his suggestions and criticism given during the course of this investigation.

[1] Kniep, H., *Verhandl. Physikal. Med. Ges. Wurzburg.*, **46**, 1–18 (1919).

[2] It should be noted that the use of the terms "compatible" and "incompatible" refers only to the interactions of mycelia determined by the mating type factors and that fusion of hyphae may take place between any two mycelia belonging to the same species, regardless of mating type. Compatible mycelia are those which when paired produce diploid mycelium bearing true clamp connections, each cell of the diploid mycelium containing a dicaryon.

[3] Buller, A. H. R., *Nature*, **126**, 686–689 (1930).

[4] Vandendries, R., and Brodie, H. J., *La Cellule*, **42**, 165–210 (1933).

[5] Dickson, H., *Ann. Bot.*, **49**, 181–204 (1935).

[6] Brodie, H. J., *Am. J. Bot.*, **35**, 312–320 (1948).

[7] Brunswik, H., *Z. Botan.*, **18**, 481–498 (1926).

[8] Quintanilha, A., *Bol. Soc. Brotereana*, **10**, 5–48 (1935).

[9] While this paper was in preparation, an abstract of work done by Papazian[10] was published which reported the results of a study of the interactions of mycelia of *Schizophyllum commune*. Papazian's findings are very similar to what has been reported in the preceding pages, in regard to the function of the *A* and *B* factors. As far as the writer can judge from the abstract, the two investigations concerning different species are mutually confirmatory.

[10] Papazian, H., *Am. J. Bot.*, **36** (suppl.), 813 (1949).

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*THE ACTION OF ALLELIC FORMS OF THE GENE A IN MAIZE. III. STUDIES ON THE OCCURRENCE OF ISOQUERCITRIN IN BROWN AND PURPLE PLANTS AND ITS LACK OF IDENTITY WITH THE BROWN PIGMENTS**

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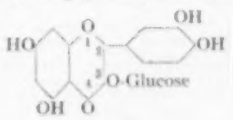
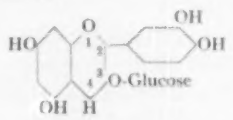
The action of the A_1 gene has long been considered the standard example in maize of a gene-controlled, unit step in biosynthesis. This view rests on the work of Sando, *et al.*, who isolated the flavonol isoquercitrin (I) from brown husks of *aa B Pl* plants[1] and obtained the anthocyanin chrysanthemin (II) from purple husks of *A B Pl* plants.[2] From these findings it has been inferred generally that the *A* gene is concerned with the reduction at the 4-position either of isoquercitrin to chrysanthemin or of some precursor which these pigments have in common.



I



II

In spite of its appeal to simplicity this interpretation is subject to several reservations. (1) Although it is apparent from superficial observation alone

that the *A* gene determines the presence of purple pigment, it is not clear that isoquercitrin, known to be present in the brown plant, is absent or reduced in the purple plant as would be expected on the current hypothesis; there is no report of an attempt to isolate isoquercitrin from the purple plant. (2) Pigmented tissues of the brown plant (*aa B Pl*) have yellowish brown cell walls whereas the cells of full-purple tissues (*A B Pl*) have purple vacuoles and colorless cell walls. From a study[3] of these plants and others carrying different intermediate alleles of *A* it was concluded that the *A* alleles are concerned with some step affecting the relative quantities of purple and brown pigments. Heretofore it has been assumed that the brown pigment is isoquercitrin but this identity is not established. (3) Since the original isolation of chrysanthemin additional purple pigments have been recovered from *A B Pl* plants.[4] Hence, even though it were established that isoquercitrin is affected by *A* action it is not valid to evaluate the action of *A* on the basis of the difference in structure between isoquercitrin and one of these purple pigments, chrysanthemin.

The present paper reports the lack of identity between the brown pigment of the *aa B Pl* plant and isoquercitrin and the occurrence of isoquercitrin in husks of purple maize.

Preliminary observations suggest that the brown pigment is not isoquercitrin. (1) Aqueous extraction of the brown husks of *aa B Pl* plants yields a deep yellowish brown solution which becomes black on treatment with dilute ferric chloride solution; analogous extracts from green husks of *A b pl* plants give only a faint olive-green color with this test. Brown pigments have been obtained free from the original extract of the brown plant and these give a black reaction with ferric chloride. However, even relatively concentrated solutions of isoquercitrin produce only an olive-green color with this reagent. (2) In dilute acid the brown pigment retains a strong yellowish brown color; on the other hand acid solutions of isoquercitrin are pale yellow to colorless. (3) The separated pigment from the brown plant undergoes a reversible change from yellowish brown in acid solution to deep reddish brown in basic media; in contrast, the nearly colorless acid solution of isoquercitrin changes to bright yellow on addition of base.

A method has been devised which separates the brown and yellow pigments. This technique, hereafter referred to as the "phase test," has much in common with the separation of phenols and certain organic acids by partition between ether and a water solution of a metal salt of a weak acid; this procedure, which has been modified somewhat since it was found necessary to use ethyl alcohol in place of ether, is as follows:

The aqueous extract of husks is treated with an equal volume of ethyl alcohol (95%) in a test-tube. Successive portions of anhydrous potassium carbonate are added ac-

companied with slight agitation until the alcohol and water layers separate (under standard conditions 7 g. of carbonate per 10 ml. of extract are employed).

When this test is applied to the extract of brown *aa B Pl* plants the alcohol layer is bright yellow whereas the water layer is deep reddish brown. Since it might be argued that the pigment in the alcohol layer is the same as that in the water layer but present in lower concentration, it is significant that under the conditions of this test the distributions of the pigments to the respective layers are complete. That brown pigment is absent in the alcohol layer of this test is indicated by the behavior of several different brown pigments which have been isolated from extracts of *aa B Pl* plants according to procedures[5] which do not remove isoquercitrin; in independent phase tests each of these pigments is distributed completely to the water layer, the respective alcohol layers remaining colorless. Furthermore, the reactions of the yellow alcohol layer to color tests are negative for the brown pigments and are identical with those of isoquercitrin. Finally, the localization of isoquercitrin in the alcohol layer in these phase tests is supported by the behavior of a sample of crystalline isoquercitrin[6] which under identical test conditions distributes to the alcohol phase.

An attempt has been made to obtain spectrophotometric evidence of the presence of isoquercitrin in the alcohol layer of the phase test of plant extracts. From these efforts it is apparent that there are other substances present in this layer which absorb in the ultra-violet where isoquercitrin has its characteristic bands. Independent studies indicate that at least one of these substances is a flavonol distinct from isoquercitrin. In view of this difficulty separation by paper chromatography[7] was attempted. This technique has been employed recently in the separation of water-soluble plant pigments[8] and of mixtures of pure flavonols.[9] In adapting it to the isolation and subsequent spectrophotometric analysis of the pigments in the alcohol layer of the phase test the following procedure was employed:

Ten ml. of the original aqueous extract of husk tissue is diluted with an equal volume of 95% ethyl alcohol and the phases induced by addition of potassium carbonate. The alcohol layer is removed, centrifuged and neutralized with hydrochloric acid. It is then evaporated at room temperature and the pigment taken up in ethyl acetate leaving the neutralization salt behind. After evaporation of the ethyl acetate the pigment is taken up in a minimal quantity of a solution of water and 95% ethyl alcohol (1:3 volumes, respectively) which carries 1% HCl and is spotted 4 cm. from one end of a strip of Whatman No. 1 filter paper having the dimensions 5 cm. × 46 cm. In order to obtain an initially narrow band spotting was effected with the edge of a microscope slide. The paper is suspended in a cylindrical hydrometer jar over a solution of butanol-acetic acid-water solvent (40:10:50 volume ratio). After 5 or 6 hrs. the strips are lowered to contact the solvent layer and movement of the solvent allowed to proceed from 24 to 48 hrs. (solvent travels from 30 to 40 cm.). Ordinarily two pigment solutions were partitioned on each strip, each jar accommodating three strips. To check the position of isoquercitrin on the test papers, a control paper spotted with the pure pigment was included in

each chamber. After air drying, the bands of pigment were developed with ammonia vapor, marked under ultra-violet light and cut from the strip. Pigments were eluted in 0.5% hydrochloric acid at room temperature and absorption curves obtained with a Beckman Model DU Quartz Spectrophotometer.

This procedure was followed in the analysis of brown and purple husks of individual maize plants. A comparison of the R_f values (ratio of distance traveled by the pigment on the paper strip to that traveled by the solvent) of pure isoquercitrin and the corresponding pigments from the tested plants (table 1) gives strong indication that both brown and purple

TABLE 1

THE R_f VALUES OF PURE ISOQUERCITRIN AND THE CORRESPONDING YELLOW PIGMENT IN EXTRACTS OF BROWN (*aa B Pl*) AND PURPLE (*A B Pl*) PLANTS

Chromatogram series[a]	Source or plant no.	Phenotype	R_f
17	Isoquercitrin	...	0.66
17	49-923.1-1	Brown	0.68
17	49-923.1-2	Brown	0.69
17	49-923.1-3	Brown	0.68
17	49-923.1-4	Brown	0.68
18	Isoquercitrin	...	0.80
18	49-923.2-1	Purple	0.77
18	49-923.2-2	Purple	0.77
18	49-923.2-3	Purple	0.78
18	49-923.2-4	Purple	0.78
19	Isoquercitrin	...	0.80
19	49-227.1-1	Brown	0.81
19	49-227.2-1	Purple	0.80
19	49-227.2-2	Purple	0.80
23	Isoquercitrin	...	0.69
23	49-231.2-5	Brown	0.74
23	49-231.2-9	Brown	0.74
23	49-231.3-1	Purple	0.73
23	49-231.3-3	Purple	0.73
34	Isoquercitrin	...	0.60
34	45-545.2-3	Purple	0.64
34	45-545.2-6	Purple	0.63
34	45-545.2-8	Purple	0.62
34	45-545.2-10	Purple	0.63

[a] Each series comprises chromatograms developed in a single chamber and simultaneously.

plants contain isoquercitrin. Chromatograms of these plant extracts may show two additional bands of pigment which behave like isoquercitrin in that they give a yellow color with ammonia. One of these has an R_f of 0.75 that of isoquercitrin; the other occurs at or near the solvent front. Both are absent from control chromatograms of isoquercitrin.

The spectral absorption curve of pure isoquercitrin taken up in alcohol

and measured directly (curve 1, Fig. 1 *A*) has peaks at or close to 2575 A and 3625 A; minima at 2375 A and 2825 A; and a characteristic sloping plateau from 2950 A to 3150 A. Curve 2, figure 1 *A*, of isoquercitrin from a control chromatogram initially spotted with a concentrated solution of

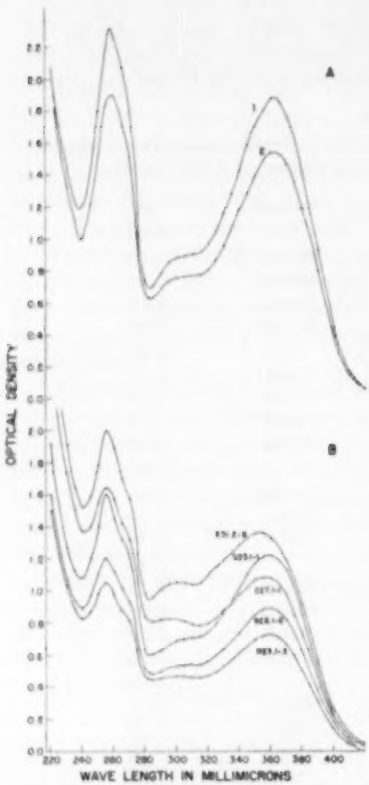


FIGURE 1

Spectral absorption curves of pigments following elution from paper chromatograms. (*A*) Curve 1, isoquercitrin measured directly; curve 2, isoquercitrin from control paper chromatogram. (*B*) The corresponding yellow pigments from chromatograms of preparations from the indicated brown (*aa B Pi*) plants. Pigments eluted and measured in 0.5% hydrochloric acid in 95% ethyl alcohol.

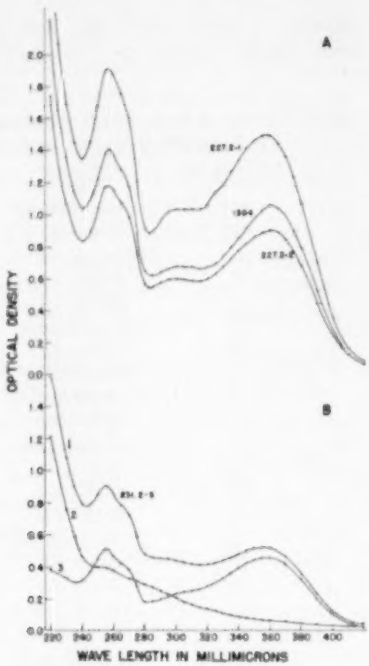


FIGURE 2

Spectral absorption curves of (*A*) the yellow pigments corresponding to isoquercitrin from chromatograms of preparations from the indicated purple (*A B Pl*) plants. (*B*) Curve 1, the yellow pigment corresponding to isoquercitrin from chromatogram of extract from the indicated brown plant; curve 2, the paper impurity eluted from a non-pigmented portion of the same chromatogram (see text for details); curve 3, plot of the differences in density values between curves 1 and 2. Pigments eluted and measured in 0.5% hydrochloric acid in 95% ethyl alcohol.

the pure pigment, is similar to curve 1, indicating that the absorption properties of the pigment are not modified by the paper partition method. Absorption curves of the yellow pigments from extracts of five brown (*aa B Pl*) plants are reproduced in figure 1 *B*. In each case the measurement was made on a solution obtained by elution of the yellow band having an R_f value similar to that of isoquercitrin on a corresponding control chromatogram. The presence of isoquercitrin in these plants is indicated by the close similarity of these curves to that of the pure pigment. However, there are several notable deviations: in the cases of the curves from the plants the absorption near 2200 A is relatively much greater, there is a tendency for both maxima to be displaced toward the shorter wave-lengths, for the minima to be displaced toward the longer wave-lengths and, instead of the gently sloping plateau in the 2950 A to 3150 A range, some of these curves exhibit a slight peak in this region. These deviations are explained if it is assumed that the measured solutions carried, in addition to isoquercitrin, a substance whose absorption is great in the 2200 A region and diminishes steadily on approaching the visible range. That such a substance is present on the paper chromatograms is indicated by the curves in figure 2 *B*. Here the uppermost curve comes from an elution of that portion of a paper chromatogram carrying the isoquercitrin band from the indicated brown plant; in general character it resembles that of pure isoquercitrin but deviates from it in the same direction as, and to an even greater extent than the curves in figure 1 *B*. A portion of this same paper strip taken from 3 cm. below the isoquercitrin band in a region which gave no fluorescence with ultra-violet light was removed. This blank piece was equal in size to that which gave curve 1, the two being handled simultaneously and under identical conditions. The solution obtained by extraction of this non-pigmented portion of the paper gave curve 2, figure 2 *B*. On the assumption that the paper impurity is present throughout the chromatogram and was present in the isoquercitrin band also, it is valid to plot a corrected curve for isoquercitrin from the differences between the density values of curves 1 and 2. This adjusted curve (Fig. 2 *B*, curve 3) is similar to that expected for isoquercitrin.

As noted in table 1, on each chromatogram of the purple (*A B Pl*) plants there is a yellow band corresponding to isoquercitrin. It is significant that in all cases these bands are much less intense than those from the brown plants suggesting that less of this pigment occurs in purple plants. This is borne out by the absorption curves obtained from these bands. In all tested cases (six bands were analyzed) extremely low density values were obtained and the curves resembled that of the paper impurity (Fig. 2 *B*) giving only slight indication of the presence of isoquercitrin. By an alternate procedure larger amounts of isoquercitrin were obtained for initial spotting on the papers:

Fifty ml. of the original aqueous extracts of purple husks were extracted exhaustively with ether. After evaporation of the ether portions the residue was taken up in the aqueous-alcoholic spotting solvent and the chromatograms developed as described previously.

The absorption curves of the yellow bands from three purple plants obtained following this procedure are reproduced in figure 2 *A*. Clearly, these indicate the presence of isoquercitrin in the purple plant.

The solutions which gave the spectral absorption curves typical for isoquercitrin in figure 1 *B* were prepared from the alcohol layer of the phase test of brown plant extracts. Since the brown pigments are excluded from this layer it may be concluded that these are distinct from isoquercitrin. This distinction makes it necessary to take account of the brown pigments in any scheme representing *A* action, since it is known that the extent of their occurrence is influenced by the *A* gene present.

While the evidence presented here indicates that isoquercitrin is present in both brown (*aa B Pl*) and purple (*A B Pl*) plants it is not reasonable to conclude that the *A* alleles do not affect this pigment, since there is strong indication that less of it is present in plants of the latter type. Nevertheless, since the *A* gene is now known to affect the synthesis of a number of related chemical substances (viz., the brown pigments, and the purple pigments in addition to chrysanthemin) there is little basis for the generally accepted view that the action of this gene is concerned solely if at all with the oxidation-reduction step which makes the difference between isoquercitrin and chrysanthemin.

[*] A portion of the work reported here was conducted at the University of Missouri.

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[5] The investigations of the brown pigments and their significance from the standpoint of action of the *A* gene will be reported elsewhere.

[6] This sample traces to the original pigment isolated from brown maize by Sando *et al.* Its receipt from Prof. M. M. Rhoades, who obtained it from the late Prof. R. A. Emerson, is gratefully acknowledged.

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DETERMINATION OF THE SPECTRUM OF THE FLOW OF BROWNIAN MOTION

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1. Brownian Motion.—The Brownian motion of N. Wiener[3] can be defined as follows: Let $(\Omega, \mathfrak{F}, Pr)$ be a *probability space*, i.e., $\Omega = \{\omega\}$ is a set of elements ω, $\mathfrak{F} = \{E\}$ is a Borel field of subsets E of Ω, and $Pr(E)$ is a countably additive measure defined on $\mathfrak{F}$ satisfying the condition $Pr(\Omega) = 1$. Let further $S = \{s \mid -\infty < s < \infty\}$ be a one-dimensional Euclidean space, and let $\mathfrak{I} = \{I\}$ be the family of all finite intervals $I = (a, b) = \{s \mid a < s \leqq b\}$. We denote by $|I|$ the length $b - a$ of the interval $I = (a, b)$, and $I = I_1 + I_2$ means that I is a union of two intervals I_1 and I_2 disjoint from each other.

A real-valued function $x(I, \omega)$ defined for $I \in \mathfrak{I}$ and $\omega \in \Omega$ is a *Brownian motion* if the following conditions (A), (B), (C) are satisfied:

(A) For any fixed $I \in \mathfrak{I}$, $x(I, \omega)$ is a measurable function of ω on Ω and has a Gaussian distribution with mean value 0 and variance $\sigma = |I|$, i.e.,

$$E_{I,\alpha,\beta} \equiv \{\omega \mid \alpha < x(I, \omega) < \beta\} \in \mathfrak{F} \tag{1}$$

$$Pr(E_{I,\alpha,\beta}) = \frac{1}{\sqrt{2\pi\sigma}} \int_\alpha^\beta e^{-u^2/2\sigma}\, du \tag{2}$$

for any $I \in \mathfrak{I}$ and for any real numbers α, β with $\alpha < \beta$.

(B). For any finite number of mutually disjoint intervals $I_k \in \mathfrak{I}$, $k = 1, \ldots, n$, the corresponding functions $x(I_k, \omega)$, $k = 1, \ldots, n$, are independent in the sense of probability, i.e.,

$$Pr(\bigcap_{k=1}^n E_{I_k,\alpha_k,\beta_k}) = \prod_{k=1}^n Pr(E_{I_k,\alpha_k,\beta_k}) \tag{3}$$

for any real numbers α_k, β_k with $\alpha_k < \beta_k$, $k = 1, \ldots, n$.

(C) $x(I, \omega)$ is additive in I, i.e.,

$$x(I, \omega) = x(I_1, \omega) + x(I_2, \omega) \tag{4}$$

almost everywhere on Ω if $I = I_1 + I_2$.

A Brownian motion $x(I, \omega)$ is *fundamental* if the following condition is satisfied:

(D) The Borel field $\mathfrak{F}'$ generated by the family $\{E_{I,\alpha,\beta} \mid I \in \mathfrak{I}, -\infty < \alpha < \beta < \infty\}$ is dense in $\mathfrak{F}$, i.e., for any $E \in \mathfrak{F}$ there exists an $E' \in \mathfrak{F}'$ such that $Pr(E \cup E' - E \cap E') = 0$.

Let $L^2(\Omega) = L^2(\Omega, \mathfrak{F}, Pr)$ be the L^2-space of all real-valued $\mathfrak{F}$-measurable functions $f(\omega)$ defined on Ω with

$$\|f\| = (\int_\Omega |f(\omega)|^2 Pr(d\omega))^{1/2} < \infty \tag{5}$$

as its norm. It is easy to see that $x(I, \omega) \in L^2(\Omega)$ for any $I \in \mathfrak{I}$. Further, for any finite number of intervals $I_k \in \mathfrak{I}$, $k = 1, \ldots, n$, the product function $\Pi_{k=1}^{n} x(I_k, \omega)$ belongs to $L^2(\Omega)$, too. Consequently, the ring $\mathfrak{R}$ generated by the functions $x(I, \omega)$, $I \in \mathfrak{I}$, is a subset of $L^2(\Omega)$. It is then easy to see that (D) is equivalent to

(D') $\mathfrak{R}$ is a dense subset of $L^2(\Omega)$, i.e., for any $f(\omega) \in L^2(\Omega)$ and for any $\epsilon > 0$ there exists a function $x(\omega)$ of the form:

$$x(\omega) = \sum_{p=1}^{q} a_p \Pi_{k=1}^{n_p} x(I_{p,k}, \omega) \tag{6}$$

such that $\|f - x\| < \epsilon$, where $I_{p,k} \in \mathfrak{I}$, $k = 1, \ldots, n_p$; $p = 1, \ldots, q$; and a_p is a real number, $p = 1, \ldots, q$.

2. *Flow of a Brownian Motion.*—Let us assume that our probability space $(\Omega, \mathfrak{E}, Pr)$ is the Lebesgue measure space (i.e., Ω is the set of all real numbers ω, $0 \leq \omega \leq 1$; $\mathfrak{E}$ is the Borel field of all Lebesgue measurable subsets E of Ω, and $Pr(E)$ is the ordinary Lebesgue measure of E with the normalization $Pr(\Omega) = 1$), or any other measure space spatially isomorphic with it.

LEMMA. *Let $x(I, \omega)$ be a fundamental Brownian motion. Then there exists a flow $\{\varphi_t\}$ defined on $(\Omega, \mathfrak{E}, Pr)$ (i.e., a one-parameter group of measure-preserving transformations $\omega' = \varphi_t(\omega)$ defined on $(\Omega, \mathfrak{E}, Pr)$ satisfying $\varphi_s(\varphi_t(\omega)) = \varphi_{s+t}(\omega)$ almost everywhere on Ω for any s and t) such that*

$$x(I + t, \omega) = x(I, \varphi_t(\omega)) \tag{7}$$

almost everywhere on Ω for any $I \in \mathfrak{I}$ and for any t $(-\infty < t < \infty)$, where $I + t = (a + t, b + t)$ is the interval obtained from $I = (a, b)$ by the translation $s \to s + t$.

This flow $\{\varphi_t\}$ is called *the flow of a Brownian motion $x(I, \omega)$*. The purpose of this note is to determine the spectral type of the corresponding one-parameter group of unitary transformations $\{U_t\}$ defined on $L^2(\Omega)$ by

$$U_t f(\omega) = f(\varphi_t(\omega)). \tag{8}$$

It was proved by N. Wiener[3] and E. Hopf[2] that $\{U_t\}$ is strongly mixing, i.e., that

$$\lim_{t \to \infty} Pr(\varphi_t(E) \cap F) = Pr(E) \cdot Pr(F) \tag{9}$$

for any $E, F \in \mathfrak{E}$, or equivalently that

$$\lim_{t \to \infty} (U_t f, g) = (f, 1) \cdot (1, g) \tag{10}$$

for any $f, g \in L^2(\Omega)$, where (f, g) denotes the inner product of f and g in $L^2(\Omega)$. Further, it was proved by H. Anzai[1] that $\{U_t\}$ has an absolutely

continuous spectrum, i.e., that, for any $f \in L^2(\Omega)$, there exists an absolutely continuous monotone non-decreasing function $\sigma(\lambda)$ defined for $-\infty < \lambda < \infty$ such that

$$(U_t f, f) = \int_{-\infty}^{\infty} e^{it\lambda}\, d\sigma(\lambda) \tag{11}$$

for any real number t, $-\infty < t < \infty$.

3. Polynomial Chaos and Spectral Decomposition of the Flow of a Brownian Motion.—In order to state our result, let us first denote by $\mathfrak{P}_n$ the set of all $x(\omega) \in L^2(\Omega)$ of the form (6) in which $n_p \leqq n$, $p = 1, \ldots, q$. $\mathfrak{P}_n$ is called the *polynomial chaos* of degree n. It is easy to see that each $\mathfrak{P}_n$ is a linear subspace of $L^2(\Omega)$ which is invariant under $\{U_t\}$, and that $\mathfrak{P}_{n-1} \subset \mathfrak{P}_n$, $n = 1, 2, \ldots$. Further, the union $\bigcup_{n=1}^{\infty} \mathfrak{P}_n$ of all $\mathfrak{P}_n$ is clearly equal to $\mathfrak{R}$ and hence is dense in $L^2(\Omega)$ by assumption. Let $\mathfrak{M}_n$ be the ortho-complement of $\bar{\mathfrak{P}}_{n-1}$ in $\bar{\mathfrak{P}}_n$, where $\bar{\mathfrak{P}}_{n-1}$ and $\bar{\mathfrak{P}}_n$ denote the closures of $\mathfrak{P}_{n-1}$ and $\mathfrak{P}_n$, respectively. Then it is easy to see that each $\mathfrak{M}_n$ is a closed linear subspace of $L^2(\Omega)$, invariant under $\{U_t\}$; that $\mathfrak{M}_n$, $n = 0, 1, 2, \ldots$, are mutually orthogonal and span the whole space $L^2(\Omega)$.

On the other hand, let $S^n = S \times \ldots \times S$ (n times) be the n-dimensional Euclidean space, and let $L^2(S^n)$ be the L^2-space of all real-valued Lebesgue measurable functions $f(s_1, \ldots, s_n)$ defined on S^n with

$$\|f\| = \left(\int_{-\infty}^{\infty} \ldots \int_{-\infty}^{\infty} |f(s_1, \ldots, s_n)|^2 ds_1 \ldots ds_n\right)^{1/2} < \infty \tag{12}$$

as its norm, where the integral is the ordinary n-dimensional Lebesgue integral. It is clear that

$$V_t^{(n)} f(s_1, \ldots, s_n) = f(s_1 + t, \ldots, s_n + t) \tag{13}$$

defines a unitary transformation $V_t^{(n)}$ of $L^2(S^n)$ onto itself. Let further $L_0^2(S^n)$ be a subspace of $L^2(S^n)$ consisting of all $f(s_1, \ldots, s_n) \in L^2(S^n)$ which are symmetric functions of $s_1, \ldots, s_n$. $L_0^2(S^n)$ is clearly a closed linear subspace of $L^2(S^n)$ and is invariant under $\{V_t^{(n)}\}$.

Then our main result can be stated as follows:

THEOREM. *$L^2(\Omega)$ can be decomposed into a direct sum of a countable infinite number of mutually orthogonal closed linear subspaces $\mathfrak{M}_n$, $n = 0, 1, 2, \ldots$, in such a way that (i) each $\mathfrak{M}_n$ is invariant under $\{U_t\}$ and (ii) $\{U_t\}$ on $\mathfrak{M}_n$ is spectrally isomorphic with $\{V_t^{(n)}\}$ on $L_0^2(S^n)$.*

The last statement of this theorem means that it is possible to find a linear isometric mapping W_n of $\mathfrak{M}_n$ onto $L_0^2(S^n)$ which satisfies $W_n^{-1} V_t^{(n)} W_n = U_t$ for any real number t, $-\infty < t < \infty$.

4. Hermite Polynomials.—The proof of our theorem can be carried out by using the Hermite polynomials $H_n(u, \sigma)$ with a parameter $\sigma > 0$, which are defined by the following formula:

$$H_n(u, \sigma) = (-1)^n (n!)^{-1} \sigma^n e^{u^2/2\sigma} \frac{d^n}{du^n} \left(e^{-u^2/2\sigma}\right) \tag{14}$$

$-\infty < u < \infty$, $\sigma > 0$, $n = 0, 1, 2, \ldots$. $H_n(u, \sigma)$ is clearly a polynomial of degree n in u and it is easy to see that the coefficient of u^n is $(n!)^{-1}$. It is well known that, for any fixed $\sigma > 0$, $H_n(u, \sigma)$, $n = 0, 1, 2, \ldots$, form an orthogonal system in the infinite interval $-\infty < u < \infty$ with respect to a weight function $\exp(-u^2/2\sigma)$, or more precisely

$$\int_{-\infty}^{\infty} H_m(u, \sigma)H_n(u, \sigma)e^{-u^2/2\sigma}\,du = \begin{cases} 0, & m \neq n, \quad m \geq 0, \quad n \geq 0, \\ \sigma^n n!, & m = n \geq 0. \end{cases} \tag{15}$$

Further, it is easy to see that $H_n(u, \sigma)$ satisfies the following functional relation:

$$H_n(u + v, \sigma + \tau) = \sum_{k=0}^{n} H_k(u, \sigma) \cdot H_{n-k}(v, \tau) \tag{16}$$

for $-\infty < u, v < \infty$ and $\sigma, \tau > 0$.

5. Homogeneous Polynomial Chaos.—Let us now consider the set $\mathfrak{C}_n$ of all $x(\omega) \in L^2(\Omega)$ of the form:

$$x(\omega) = \sum_{(n_1, \ldots, n_p)} a_{n_1, \ldots, n_p} \prod_{k=1}^{p} H_{n_k}(x(I_k, \omega), \sigma_k), \tag{17}$$

where $\{I_k\}$ $k = 1, \ldots, p\}$ is any finite system of mutually disjoint intervals from $\mathfrak{I}$; $\sigma_k = |I_k|$, $k = 1, \ldots, p$; $a_{n_1, \ldots, n_p}$ is a real number for each $(n_1, \ldots, n_p)$, and $\sum_{(n_1, \ldots, n_p)}$ denotes the sum for all p-systems of non-negative integers $(n_1, \ldots, n_p)$ such that $n_1 + \ldots + n_p = n$. $\mathfrak{C}_n$ is called the *homogeneous polynomial chaos* of homogeneous degree n.

It is not difficult to see that each $\mathfrak{C}_n$ is a linear subspace of $L^2(\Omega)$ which is invariant under $\{U_t\}$, that any two $\mathfrak{C}_m$, $\mathfrak{C}_n (m \neq n)$ are orthogonal to each other and that $\mathfrak{P}_n = \mathfrak{C}_0 + \mathfrak{C}_1 + \ldots + \mathfrak{C}_n$, for $n = 0, 1, 2, \ldots$. From this follows easily that $\overline{\mathfrak{P}}_n = \overline{\mathfrak{C}}_0 + \overline{\mathfrak{C}}_1 + \ldots + \overline{\mathfrak{C}}_n$ and $\mathfrak{M}_n = \mathfrak{P}_n$, $n = 0, 1, 2, \ldots$, where we denote by $\overline{\mathfrak{P}}_n$, $\overline{\mathfrak{C}}_n$ the closures of $\mathfrak{P}_n$, $\mathfrak{C}_n$, respectively.

Let us now put $f = W_n x$ if $x(\omega) \in \mathfrak{C}_n$ is of the form (17) and if $f(s_1, \ldots, s_n) \in L_0^2(S^n)$ is of the form:

$$f(s_1, \ldots, s_n) = (n!)^{-1/2} \sum_{(n_1, \ldots, n_p)} a_{n_1, \ldots, n_p} \sum_{(J)} \prod_{i=1}^{n} \chi_{J_i}(s_i), \tag{18}$$

where $\chi_J(s)$ is the characteristic function of a set J; $(J_1, \ldots, J_n)$ is a permutation of $(I_1, \ldots, I_1$ (n_1 times), $I_2, \ldots, I_2$ (n_2 times), $\ldots, I_p, \ldots, I_p$ (n_p times)); $\sum_{(J)}$ means the sum for all possible different permutations of $(J_1, \ldots, J_n)$ (note that there are $n!/n_1! \ldots n_p!$ different permutations); $a_{n_1, \ldots, n_p}$ is a real number for each $(n_1, \ldots, n_p)$ and $\sum_{(n_1, \ldots, n_p)}$ denotes the same thing as in (17). In other words, (18) means that $f(s_1, \ldots, s_n) = (n!)^{-1/2} a_{n_1, \ldots, n_p}$ if all s_i, $i = 1, \ldots, n$, belong to $\bigcup_{k=1}^{p} I_k$ and

if the number of elements s_i which belong to I_k is n_k for $k = 1, \ldots, p$. Otherwise (i.e., if at least one element s_i, $1 \leqq i \leqq n$, does not belong to $\bigcup_{k=1}^{p} I_k$), $f(s_1, \ldots, s_n) = 0$.

It is then easy to see that W_n is a linear transformation of $\mathfrak{Q}_n$ onto a dense linear subspace of $L_0^2(S^n)$ which we shall denote by $\mathfrak{Q}_n'$. It is also easy to see that

$$\|x\|^2 = \sum_{(n_1, \ldots, n_p)} |a_{n_1, \ldots, n_p}|^2 \prod_{k=1}^{p} \frac{\sigma_k^{n_k}}{n_k!} \tag{19}$$

if $x(\omega) \in \mathfrak{Q}_n$ is of the form (17) and that

$$\|f\|^2 = (n!)^{-1} \sum_{(n_1, \ldots, n_p)} |a_{n_1, \ldots, n_p}|^2 \frac{n!}{n_1! \ldots n_p!} \prod_{k=1}^{p} \sigma_k^{n_k} \tag{20}$$

if $f(s_1, \ldots, s_n) \in \mathfrak{Q}_n'$ is of the form (18). This shows that W_n is an isometric transformation of $\mathfrak{Q}_n$ onto $\mathfrak{Q}_n'$. Further it is clear from the definition of U_t, $V_t^{(n)}$ and W_n that $U_t = W_n^{-1} V_t^{(n)} W_n$ on $\mathfrak{Q}_n$ for any t, $-\infty < t < \infty$. Since $\mathfrak{Q}_n$ and $\mathfrak{Q}_n'$ are dense in $\mathfrak{M}_n$ and $L_0^2(S^n)$, respectively, it is possible to extend W_n to a linear isometric transformation (which we shall again denote by W_n) of $\mathfrak{M}_n$ onto $L^2(S^n)$ which satisfies $U_t = W_n^{-1} V_t^{(n)} W_n$ on $\mathfrak{M}_n$ for any t, $-\infty < t < \infty$. This completes the proof of the Theorem.

It is easy to see that the results of Wiener, Hopf and Anzai stated in § 2 follow immediately from our Theorem. The decomposition of $L^2(\Omega)$ into a direct sum of mutually orthogonal invariant linear subspaces $\mathfrak{M}_n$, $n = 0, 1, 2, \ldots$, on each of which our $\{U_t\}$ behaves very nicely, makes the situation very simple and easy to handle. The notion of polynomial chaos $\mathfrak{P}_n$ and the discovery of its usefulness in our problem of spectral analysis are due to N. Wiener.[3] Wiener's original argument was more complicated since he discussed only $\mathfrak{P}_n$ and not $\mathfrak{M}_n$ nor $\mathfrak{Q}_n$. The idea of taking the ortho-complement $\mathfrak{M}_n$ of $\mathfrak{P}_{n-1}$ in $\mathfrak{P}_n$ is due to J. von Neumann (oral communication). The introduction of Hermite polynomials $H_n(u, \sigma)$ with a parameter $\sigma > 0$ satisfying the functional relation (16), and the definition (17) of homogeneous polynomial chaos $\mathfrak{Q}_n$ in terms of these Hermite polynomials $H_n(u, \sigma)$ made it easy to set up an isomorphism between $\mathfrak{M}_n$ and $L_0^2(S^n)$ which carries $\{U_t\}$ over into $\{V_t^{(n)}\}$. Similar results were also obtained recently by K. Ito in a different way by using the method of stochastic integral.

The detail of the proof and the discussion of allied problems are left to a subsequent paper where a more general class of Brownian motions will be treated.

[1] Anzai, H., "A Remark on Spectral Measures of the Flow of Brownian Motion," *Osaka Math. J.*, **1** 95–97 (1949).

[2] Hopf, E., *Ergodentheorie*, 1937. §14.

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THE CRYSTALLOGRAPHIC SYMMETRIES DETERMINABLE BY X-RAY DIFFRACTION

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The problem of how to determine the symmetry of a given crystal has been an important one from the early days of the science of crystallography. Symmetry requires the various properties of a crystal to be equivalent in symmetrically equivalent directions. Accordingly, the general nature of a test for the presence or absence of a particular kind of symmetry is to subject the possibly symmetrical directions of a crystal to some physical or chemical action and then ascertain whether the crystal's response is equivalent in these directions or not.

Unfortunately, some tests inherently involve certain symmetry themselves and this prevents one from judging whether the symmetry resulting from such a test derives from the crystal or from the test. This kind of dilemma occurs in testing the symmetry of a crystal by its diffraction effects. When a crystal diffracts x-rays whose wave-length is not in the immediate neighborhood of an absorption edge of one of the chemical elements in the crystal, the diffraction effects are inherently centrosymmetrical. This was pointed out by Friedel[1] as early as 1913, only a year after the discovery of the diffraction of x-rays by crystals made by von Laue and his students.[2, 3, 4] The cause of this lies in the impossibility of experimentally observing the phases of the x-ray diffraction spectra. Thus, if a set of planes *hkl* "reflects" x-rays with amplitude $|F_{hkl}|$ and phase φ, the set of planes $\overline{hkl}$ "reflects" with identical amplitude $|F_{hkl}|$, but with phase $-\varphi$. Since the amplitudes (in the form of intensities, or F^2's) but not the phases of the reflections can be experimentally observed, diffraction effects are inherently centrosymmetrical, and, even though a crystal does not have a center of symmetry, its diffraction effects do display a center. Therefore, although there are 32 symmetry combinations possible in crystals, only 11 symmetry combinations can be distinguished by the symmetry of their diffraction effects. None of these 11 sets uniquely characterizes any of the 32 crystal classes (i.e., the 32 combinations of symmetry possible for crystals).

One might suppose that this feature provides an ultimate limitation on the detection of symmetry by x-ray diffraction. But in 1919 Niggli[5] showed that the space group symmetry elements which involve translation components could be identified by the characteristic missing spectra which they caused to occur in the diffraction effects. Later the writer[6, 7] showed that by combining the information from Friedel's law and Niggli's "ex-

tinctions" (missing spectra), a total of 120 "diffraction groups" could be distinguished. The total number of space groups, or kinds of internal symmetry which are possible in crystals, is 230. Most of the diffraction groups include several space groups, namely, those from the several centrosymmetrical crystal classes which have the same Niggli extinctions. But 59 of these diffraction groups embrace only a single space group (counting enantiomorphous pairs as one space group) and consequently 59 out of the 219 space groups (excluding enantiomorphous duplicates) can be distinguished by a mere qualitative inspection of the diffraction data.

At the 1946 meeting of the American Society for X-Ray and Electron Diffraction, the writer announced[8] that all space groups except those pairs differing solely by an inversion could be distinguished by diffraction means provided that the spectra are considered on a quantitative basis. In the light of this discovery, Niggli's extinctions, which amounted to *intensity* = 0 for certain special spectra, were seen to be merely a qualitatively obvious special case of a much more general control of spectral intensity by the crystal symmetry. It thus became clear that the key to crystal symmetry was contained in the diffraction spectra of the crystal.

In the same paper[8] the writer showed that the key to the structure of the crystal also lay fallow in the spectra, and one device for finding the structure, namely, the *implication* method, was suggested. This was an astonishing result which was received with incredulity by nearly everyone, because it was a well-established belief[9] that, since the phases of the diffraction cannot be observed, there is not necessarily a solution for a crystal structure from diffraction data alone.

Some more recent developments make it possible to treat the determinability of crystal symmetry by x-ray diffraction data on a more straightforward basis. The electron density of a crystal at a selection of points xyz is related to the complex amplitudes, F_{hkl}, of the diffraction spectra hkl by

$$\rho(xyz) = \sum_{h}\sum_{k}\sum_{l}^{-\infty\ \infty} F_{hkl}e^{2\pi i(hx + ky + lz)}. \qquad (1)$$

Patterson[10, 11] showed that another function, not involving the complex F's, but rather their observable squares, bears a simple relation to (1). Patterson's function,

$$A(uvw) = \sum_{h}\sum_{k}\sum_{l}^{-\infty\ \infty} F^2_{hkl}e^{2\pi i(hu + kv + lw)}, \qquad (2)$$

has maxima where vectors with components u, v, w span maxima in (1). Thus, if maxima occur at $x_1y_1z_1$ and $x_2y_2z_2$ in (1), then maxima occur in (2) at $\pm uvw$, where

$$\left.\begin{aligned} u &= x_2 - x_1 \\ v &= y_2 - y_1 \\ w &= z_2 - z_1 \end{aligned}\right\}. \qquad (3)$$

Of course, maxima occur in (1) where the electron density is high, i.e., at atom locations.

The relation involved between (1) and (2) can be rendered more evident if points are substituted for atoms in the crystal structure.[12] Then (1) is transformed into a collection of points called the *fundamental set*, and (2) is transformed into another collection of points called the *vector set*. To derive the vector set from the fundamental set, the vectors between all pairs of points in the fundamental set are drawn, then the vectors are reassembled at a common origin. The collection of points at the ends of the reassembled vectors constitutes the vector set.

It is an easy matter to find the vector set for any given fundamental set. The writer has recently shown[12] that it is also possible to find the fundamental set for any vector set. But the vector set is a representation of (2), simplified so that the maxima become points,[13] and the fundamental set is a representative of (1), similarly simplified. This implies that if (2) can be solved, (1) can be derived from it. The interesting consequence of this is that, although (1) cannot be computed from diffraction data because of the unobservability of the phase component of the complex F's, (2) involves F^2's and therefore does not require a knowledge of these phase components. Therefore, in principle, crystal structures are solvable from diffraction data alone.

An immediate consequence of this conclusion is that all crystal symmetries are determinable from diffraction data, for the symmetry of a crystal is an incidental aspect of its structure. Furthermore, since the symmetry of the fundamental set and the crystal are the same, and since the fundamental set is derivable from the vector set, it follows that the key to the symmetry of the crystal is in its vector set.

Actually, vector sets display fewer possible symmetries than the fundamental sets, since the former must all be centrosymmetrical. The writer has shown another way of looking at this.[12] When the vectors between points in the fundamental set are transferred to the origin of the vector set, the translation components of the symmetry elements relating the vectors are lost to the symmetry of the vector set, although other components are retained. In this way it can be shown that, although the symmetries of the fundamental sets comprise the 230 space groups, there are only 24 space group symmetries possible in vector sets. But in this degeneration of actual symmetry, the key to the symmetry of the original fundamental set is not lost. The translation components of the vectors between all atoms in the fundamental set which are symmetrically equivalent,

when transferred to the vector set, cause a concentration of points at the ends of these vectors at loci characteristic of the lost translation. The principal key to crystallographic symmetry is these concentrations in vector space. These are discovered for any crystal by computing the full three-dimensional Patterson synthesis (2). The unaided computation of this synthesis is so laborious that it has very rarely been carried out to date. But with the recent development of the Pepinsky Fourier synthesizer,[14] this full synthesis has now become quite practical.

The writer has tabulated the symmetrical concentrations for the 230 space groups. The tabulation shows that all space groups are uniquely

TABLE 1

PAIRS OF SPACE GROUPS NOT DISTINGUISHABLE BY LOCATIONS OF SYMMETRICAL IMAGES

THE ENANTIOMORPHOUS PAIRS			
$C3_1$ $C3_2$	$P1$ $P\bar{1}$	$C\bar{6}$ $C6/m$	$I222$ $I2_12_12_1$
$C3_121$ $C3_221$	$C3$ $C\bar{3}$		$I23$ $I2_13$
$C3_112$ $C3_212$	$R3$ $R\bar{3}$		
$P4_1$ $P4_3$	$P4$ $P\bar{4}$		
$P4_12$ $P4_32$	$I4$ $I\bar{4}$		
$P4_12_1$ $P4_32_1$			
$C6_1$ $C6_5$			
$C6_2$ $C6_4$			
$C6_122$ $C6_522$			
$C6_222$ $C6_422$			
$P4_132$ $P4_332$			

characterized by these concentrations except for the pairs shown in table 1. There are four kinds of categories where the concentrations determine pairs of space groups instead of a single space group. Each category is listed as a column in table 1. Column one contains the 11 pairs of enantiomorphous space groups. Column two contains 4 pairs of space groups, each pair containing a cyclical group of the first sort and the corresponding cyclical group of the second sort. Columns three and four contain three more pairs which cannot be placed in either of the other categories.

Now, all space groups except the 19 pairs shown in table 1 can be distinguished merely by the locations of the concentrations. But by taking into account some obvious aspects of the *patterns* in the concentrations, all space groups except the 11 enantiomorphous pairs in the first column of table 1 can be distinguished. Thus, in the pairs $P4$, $P\bar{4}$ and $I4$, $I\bar{4}$ the first member of each pair contains satellites[8] while the second does not. (A satellite is merely a duplication of the pattern motif enlarged and rotate 45° in this case.) The members of the pairs are therefore easily distinguished by the qualitative appearance of the patterns in the concentration. In the first member of the pairs $I222$, $I2_12_12_1$ and $I23$, $I2_13$, the patterns in the three orthogonal (axial) planar concentrations are simple projections of one another. For the second member of the pairs, the corresponding patterns are also projections of one another, but with origins shifted $\frac{1}{4}\frac{1}{4}0$ etc. The members of these pairs can therefore also be distinguished by the qualitative appearance of the patterns in the concentrations. It remains to distinguish between members of the three pairs $P1$, $P\bar{1}$; $R3$, $R\bar{3}$; and $C3$, $C\bar{3}$. These can be distinguished if it is possible to distinguish points of weight 1 and weight 2, since the first and second members of these pairs differ in exactly this way.[8] This can readily be done provided that the x-ray diffraction data from which the Patterson synthesis (2) has been made is on an absolute basis. Then, the total number of electrons within each peak is of the form z_1z_2 for the first member of each pair and $2z_1z_2$ for the second member of each pair, where z_1 and z_2 are the atomic numbers of atoms 1 and 2 in the crystal structure. For peaks in the symmetrical concentration loci of $R3$, $R\bar{3}$, $C3$ and $C\bar{3}$, this condition reduces to peaks of z^2 and $2z^2$, where z is the atomic number of an atom in the structure.

Thus, an incidental consequence of the conclusion that crystal structures are solvable from diffraction data is that all space groups can be distinguished from diffraction data except that one cannot distinguish between members of the enantiomorphous pairs. A rather similar but less general conclusion has been reached on other grounds. Wilson[15] has recently shown that the presence or absence of an inversion center (not detectable by observation of relative intensities, which results only in distinguishing the 11 centrosymmetrical crystal classes) can be discerned by a quantitative statistical analysis of all spectra produced by the crystal. Rogers[16] then pointed out that this additional feature could be used to modify the writer's diffraction symbols. When the possibility of distinguishing centrosymmetrical zones is taken into account, this permits distinguishing all but the 11 enantiomorphous pairs and the following four pairs of space groups:

$$\left\{\begin{matrix} I222 \\ I2_12_12_1 \end{matrix}\right. \quad \left\{\begin{matrix} I23 \\ I2_13 \end{matrix}\right. \quad \left\{\begin{matrix} C3\text{lm} \\ C3\text{ml} \end{matrix}\right. \quad \left\{\begin{matrix} C\bar{3}\text{lm} \\ C3\text{ml} \end{matrix}\right.$$

Since these four pairs *can* be distinguished by the vector set method, the latter is more powerful. Of course, it has this greater power because the patterns themselves are examinable in the concentration loci, and because the loci are correlated with the lattice (which accounts for distinguishing the last two pairs above).

[1] Friedel, G., "Sur les symétries cristallines que peut révéler la diffraction des rayons Röntgen," *Compt. rend.*, **157**, 1533–1536 (1913).

[2] Friedrich, W., Knipping, P., and Laue, M., "Interferenz-Erscheinungen bei Röntgenstrahlen," *Sitzber. math.-physik. Klasse Akad. Wiss. München*, **1912**, 303–322.

[3] Laue, M., "Eine quantitative Prüfung der Theorie für die Interferenz-Erscheinungen bei Röntgenstrahlen," *Ibid.*, **1912**, 363–373.

[4] Laue, M., "Röntgenstrahlinterferenzen," *Physik. Z.*, **14**, 1075–1079 (1913).

[5] Niggli, Paul, *Geometrische Kristallographie des Discontinuums*, Gebrüder Burntraeger, Leipzig, 1919, pp. 482–503.

[6] Buerger, M. J., "The Application of Plane Groups to the Interpretation of Weissenberg Photographs," *Z. Krist.*, **A91** (1935), especially pp. 287–288.

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[9] Buerger, M. J., reference 7, p. 2.

[10] Patterson, A. L., "A Fourier Series Method for the Determination of Interatomic Distances in Crystals," *Phys. Rev.*, **46**, 372–376 (1934).

[11] Patterson, A. L., "A Direct Method for the Determination of the Components of Interatomic Distances in Crystals," *Z. Krist.*, **A90**, 517–542 (1935).

[12] Buerger, M. J., "Vector Sets," *Acta Cryst.*, **3**, 87–97 (1950).

[13] It is possible to derive a vector set from any (2) (or to derive the fundamental set from (1)) by a pin-point-focussing process. To do this each F^2_{hkl} is divided by $f^2_{1,hkl}$ (or each F_{hkl} is divided by $f_{1,hkl}$), where $f_{1,hkl}$ is the scattering power of atom 1, selected to be pinpointed for spectrum *hkl*, at the temperature of the experimental determination of F^2_{hkl}.

[14] Pepinsky, R., "An Electronic Computor for X-ray Crystal Structure Analyses," *J. Applied Phys.*, **18**, 601–604 (1947).

[15] Wilson, A. J. C., "Possible X-ray Determination of a Center of Symmetry," *Research*, **2**, 246 (1949).

[16] Rogers, D., "Determination of Space Groups by Intensity Statistics, *Ibid.*, **2**, 342–343 (1949).

THE PHOTOGRAPHY OF ATOMS IN CRYSTALS

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Boersch[1] first called attention to the possibility of producing images of the atoms in crystals by using the diffraction from a grating corresponding to the reciprocal lattice. Shortly thereafter, Bragg[2] made a photograph by this method of the atoms in the crystal structure of the mineral diopside. Unfortunately, Bragg's "x-ray microscope" could only be applied to photographing that very limited class of crystal structures all of whose diffraction spectra had the same phase.

The writer immediately pointed out[3, 4] that there exists a very large class of much-used two-dimensional Fourier summations in which the phases of all coefficients are identical, and that therefore these summations could be produced optically by diffraction from a weighted reciprocal lattice, the weighting being the Fourier coefficients. The new class of syntheses for which the optical method could be used includes the Patterson[5, 6] synthesis, $P(xy)$, and the Harker[7] zero-level synthesis, $P(xy0)$. Furthermore, such syntheses were actually carried out by this optical method. More recently, the writer has also used the optical method for producing the real part of the Fourier transforms of molecules.

In one of these papers, the writer[4] also suggested that the method could be easily extended to the general Harker synthesis, $P(xyn)$, and could be extended to the photography of all crystal structures by the use of a simple device for controlling the phases of the diffraction spectra. This requires a collection of small pieces of mica cut from a single homogeneous mica cleavage. To use this method, a mica piece is placed in the path of the rays of any spectrum which requires a phase shift. To produce a particular phase shift of angle $r \cdot 2\pi$, it is only necessary to incline the mica piece so that the optical path through it is increased over that of the surrounding air by an amount $r\lambda$, where λ is the wave-length of the light used in the experiment.

The chief difficulty in practicing this method of phase control is to find mica which will furnish a cleavage flake of uniform thickness over a sufficiently large area so that the rather large number of pieces needed to control the phases of the many spectra (say, between 100 and 400) can be cut from it. Experimental work done in 1941 confirmed that this method of phase control was indeed feasible. Mica cleavages were tested for uniformity in thickness by examining them by reflection close to a broad source of monochromatic light. This has the effect of contouring the mica with interference lines which remain unbroken in regions where the thick-

ness does not vary. By this means of examination, areas of uniform thickness are easily mapped out on any mica cleavage. The inclination required to produce any required phase shift was found for any given mica sheet by setting up a two-hole diffraction "grating" with the piece of mica to be tested over one of them. Without the mica, the two holes produce a set of interference fringes. With the mica, the maxima are shifted in accordance with the phase shift produced by the mica. The drift in maxima can thus be correlated with the angle which the mica makes with the ray, and in this way a particular mica sheet of any thickness can be calibrated for phase-shift versus inclination.

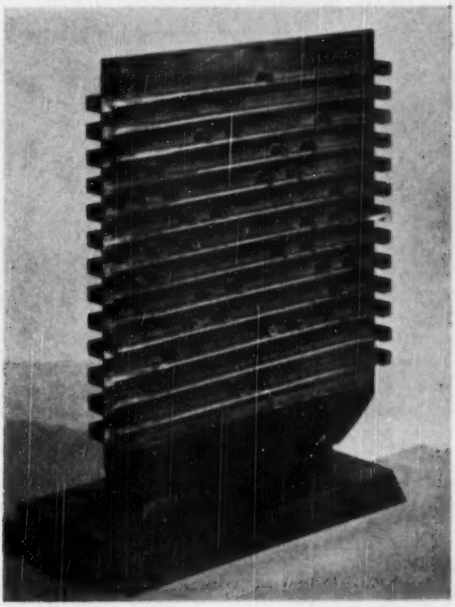


FIGURE 1

The war intervened in this experimentation, and its was discontinued until recently. In this new work, the writer was aided by a graduate student, Mr. Jay W. Lathrop, who selected and calibrated some large mica cleavages, and by Mr. John Solo, who executed the nice machining required for constructing the grating and the phase shifters.

The control of the phase of the diffraction was first applied to forming the image of the crystal structure of marcasite, FeS_2. The grating whose focused diffraction produced this image, and the method of controlling the

phases are indicated in figure 1. The grating consists of holes drilled in a metal plate at points corresponding with the points of the reciprocal lattice of marcasite, and having translations a^* and b^*. The amount of light passing through each hole of index hk is regulated by making the area of hole equal to $|F_{hk0}|$, i.e., to the absolute magnitude of the amplitude of the x-ray diffraction spectrum $hk0$ of marcasite. The phase of the light emanating from each hole in the diffraction process is regulated, if necessary, by placing an inclined mica piece, held in a metal block at the correct inclination angle, in front of the hole. Since marcasite is a centrosymmetrical crystal, the only phases which the spectra can have are 0 and π. The spectra having phase zero are not disturbed, but those having phase π

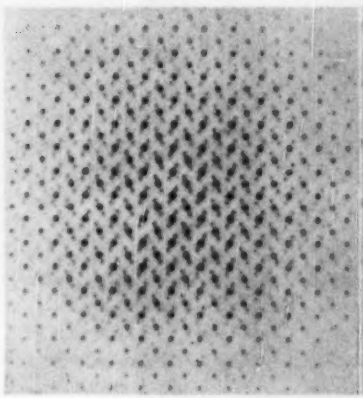


FIGURE 2

The pattern of atoms in the structure of marcasite, FeS_2, magnified 9×10^6 diameters.

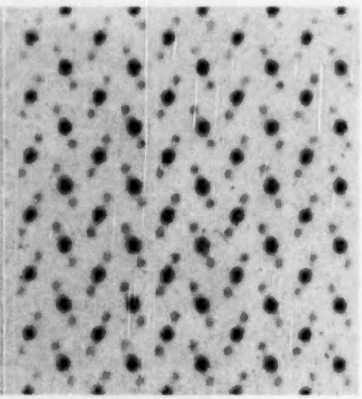


FIGURE 3

The pattern of atoms in the structure of marcasite, FeS_2, magnified 2.1×10^7 diameters.

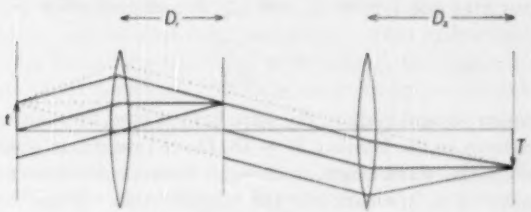
are properly phased by placing in front of each such hole a metal block. Thiry-two such blocks can be seen in figure 1. On the sloping forward face of each block there is a mica piece so set that the slope provides an extra path difference of $(2n - 1)\frac{\lambda}{2}$ for the light. This corresponds with shifting the phase of the spectrum by $(2n - 1)\pi$, which is equivalent to a phase shift of π.

The focused diffraction from this grating is shown in figures 2 and 3. Comparison of these photographs with the published diagrams of the crystal structure of marcasite[3] show that this is an accurate picture of the structure. In fact, the blackening of figures 2 and 3 is a faithful repre-

sentation of the electron density in the structure. The patterns of these photographs are characterized by circular dark areas. The darkest of these are iron atoms with 26 electrons each, and the lighter circular areas are sulfur atoms with 16 electrons each. Careful study of the photograph shows a background of faint diffraction effects, caused by the fact that a limited number of spectra were used in forming the image. This corresponds with a limitation of aperture of the lens in ordinary microscopy. The magnification of figure 2 is about 9×10^6 diameters.

Another more complicated design of phase shifter has been prepared for crystal structures lacking a center of symmetry. In this more general design, the mica sheet is mounted on a rotating device so that the angle between the mica and the rays can be set at any value required to produce any phase shift for a particular spectrum.

The whole process of image formation by using diffraction from a weighted reciprocal lattice may be looked upon as essentially a two-stage,



$$M = \frac{T}{t} = \frac{D_2\lambda_2}{D_1\lambda_1}$$

FIGURE 4

or two-wave-length, microscope. This is diagrammatically illustrated in figure 4. The diagram represents a simple optical system consisting of two lenses having focal lengths D_1 and D_2, respectively. The paths of rays ordinarily regarded as forming the image are indicated by dotted lines. But image formation can also be regarded as two diffractions in sequence. The focusing of these diffractions is illustrated by full lines. If the object is periodic (which simplifies the treatment by avoiding the explicit use of Fourier transforms) with period t, then the rays which are diffracted in the same direction by the object (i.e., rays which reach the lens as a parallel set) are focused at its right focus of the first lens to form a diffraction image. This is a collection of discrete spectra whose spacings are also periodic. This spacing is[4]

$$d^* = (D_1\lambda_1)\frac{1}{t}, \qquad (1)$$

where D_1 and λ_1 are experimental constants. The reciprocity between d^* and t causes the discrete spectra in the diffraction image to be arranged on the points of the reciprocal lattice of the crystal which is used as an object.[4]

But the spectra of the diffraction image are also sources of rays. Those which reach the second lens as a parallel set are focused by it at its right focus. The first period of the final periodic image is formed by the first-order diffraction from the diffraction image. The length of this first period[4] in the final image is

$$T = (D_2\lambda_2)\frac{1}{d^*}. \tag{2}$$

Now, the magnification of the system is

$$M = \frac{\text{image size}}{\text{object size}} = \frac{T}{t}. \tag{3}$$

Substituting for t and T from (1) and (2), this magnification is

$$M = \frac{D_2\lambda_2}{D_1\lambda_1}. \tag{4}$$

In an ordinary optical system, the same light is used throughout. In this case (4) reduces to the familiar $M = D_2/D_1$, i.e., the image:object distance ratio. But if the wave-length is changed between diffraction stages, the further factor of λ_2/λ_1 enters into the magnification. Using some reasonable values (in centimeters) of (4), a magnification of

$$M = \frac{2 \times 10^2}{5} \times \frac{5 \times 10^{-8}}{7 \times 10^{-9}} = 3 \times 10^3 \text{ diameters} \tag{5}$$

can be achieved. In fact, these are the approximate actual values used for the instruments which produced the photographs of figures 2 and 3. This great magnification is not empty, since the resolving power depends on the wave-length of the first stage and the number of spectra collected in the first diffraction image.

While x-rays cannot be focused with lenses, the x-ray diffraction produced by a crystal can be arranged in the form of a reciprocal lattice by the precession camera,[5] just as if the diffraction image were actually produced by the first lens of figure 4. The constants in the denominators of (5) represent a 5-cm. crystal-to-film distance in the precession camera, and the wave-length of Mo$K\alpha$ x-radiation. The constants in the numerator of (5) are the focal length of the lens actually used in figure 4, and the wave-length of green light. This tremendous magnification is sufficient to enlarge the image of an atom so that it would be of the order of 0.03 mm. in diameter, which is big enough so that it can be seen conveniently by a

microscope. Figures 2 and 3 were taken with a microscope of moderate magnification.

This description of microscopy can be epitomized by saying that the image is formed from the object by diffraction from the diffraction image of the object. In other words, it is two-stage diffraction. When it is caused to occur in two separate steps, with or without two different wavelengths, the phases of the diffraction spectra in the first diffraction image are lost. It is this loss of phase which the phase shifters are required to supply. If they are not supplied, a vector map of the object results instead of a simple image. It is possible to decompose a vector map[10] into simple images, and when this is done, the phases required for the phase shifters may be learned.

This generalized theory of microscopy as two stages of diffraction is not new. It was developed by the writer in 1939, in connection with the experimental development of the instrument, and was first presented at the January 10, 1941, meeting of the New York Academy of Sciences, again at the October, 1942, meeting of the Rochester Section of the American Optical Society, and on some other occasions. What makes the matter of interest now is that it is a novel way of looking at the meaning of photographs of atoms in crystals, such as those shown in figures 2 and 3.

[1] Boersch, H., "Zur Bilderzeugung im Mikroskop," *Z. tech. Physik*, 337–338 (1938), especially footnote 3, p. 338.

[2] Bragg, W. L., "A New Type of 'X-ray Microscope,'" *Nature*, **143**, 678 (1939).

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